EFFECT OF LAND MANAGEMENT PRACTICES ON PHYSICAL PROPERTIES OF SOIL AND WATER PRODUCTIVITY IN WHEAT-MAIZE SYSTEM OF NORTHWEST INDIA

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Abstract. Decline in soil physical health and crop productivity are the major concerns in wheat-maize cropping system of northwest India. Depending on objective, conservation and deep tillage are the potential solutions. Therefore, a field study was conducted to investigate the interactive effects of land management practices and irrigation regimes on soil properties and wheat-maize productivity. The tillage treatments comprised of no-tillage, strip tillage, conventional tillage and deep tillage, while the irrigation regimes included three levels based on irrigation water over pan evaporation (IW/PAN-E) ratios i.e. $I_{0.6}$, $I_{0.9}$ and $I_{1.2}$ for both wheat and maize crops. By shattering soil up to 45 cm depth, the deep tillage helped in improving infiltration rate by 31 % over conventional tillage. However, the no-tillage improved soil aggregation by 38 % than deep tillage. At subsurface depth (15-30 cm) the soil bulk density and penetration resistance were found to be significantly lower under deep tillage (1.52 Mg m^{-3} and 1.9 MPa) than conventional tillage (1.68 Mg m^{-3} and 2.8 MPa), respectively. The deep tillage enhanced root proliferation by increasing root length density by 44 % and 34 % than conventional tillage in maize and wheat, respectively. The grain yields (Mg ha^{-1}) of maize and wheat were 14 % and 12 % more under deep tillage than conventional tillage, respectively. The water productivity varied significantly under different irrigation regimes and was found to be highest at $I_{0.9}$ in maize and $I_{1.2}$ in wheat.
Keywords: *soil compaction, mechanical resistance, water transmission, root proliferation, crop productivity*

Introduction

Alternate tillage practices and excessive use of machinery leads to soil compaction and hard pan formation in subsurface soil layer (Kukal and Aggarwal, 2003; Singh et al., 2009) this may affect the growth of wheat and maize due to reduced water transmission and root proliferation (Gajri et al., 1994). The deep tillage (DT) enhances rooting system by lowering soil strength which helps in better uptake of water and nutrients from the deeper layers (Gajri et al., 1994). On the other hand, conservation tillage i.e. no-tillage with residue retention (NT) provide favorable soil environment by improving soil water transmission, moisture storage, aggregation, carbon sequestration and crop productivity (Tessier et al., 1990). This practice also improves economic performance, energy use efficiency and reduces production risks (Zentner et al., 2002; Lal, 2003). The area under NT has been substantially increased in South Asia and particularly Indo-Gangetic plains (Derpsch et al., 2010). Continuous use of NT can cause measurable changes in soil hydrological, mechanical, physical, chemical and biological properties (Lal and Elder, 2008). The other conservation tillage practice i.e. strip till (ST) disturbs the soil minimally and it also manages to keep the previous crop residue in between the rows. However, the conventional tillage (CT) practices lead to breakdown of soil structure which subsequently affects soil water transmission characteristics, soil organic matter depletion, microbial activity and crop productivity

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(Ramos et al., 2011). The CT, including intensive soil cultivation and crop residue removal and burning, have exacerbated soil erosion and degradation, thus contributing to the development of soils with low organic matter contents and a fragile physical structure. There is thus an urgent need to identify land management practice which would help in sustaining soil physical health as well as crop productivity. Since, it is difficult to sow wheat and maize directly in the standing stubbles and the loose residue of previous crops, the farmers, mostly, burn them for seed bed preparation. The crop residue burning or removal can cause huge loss to soil fertility, physical health and environmental issues such as aerosols (Sidhu et al., 2007). However, with the help of NT practices, residue management problem can be solved to a great extent (Afzalinia and Zabihi, 2014). Water stress (both due to excessive and deficient soil moisture conditions) at any growth stage of wheat and maize can reduce crop and water productivity (Paudyal et al., 2001). Thus, optimum irrigation is a solution to this problem. The knowledge of wheat and maize performance at various irrigation regimes is of utmost importance in a semi-arid environment to improve water productivity. Water scarcity and uneven distribution are considered to be the primary limiting factors affecting wheat and maize production in the semiarid region (Kang et al., 2002; Wang et al., 2009). There are few reports on the interactive effects of straw mulching and DT in relation to irrigation regimes on crop yield. There is thus, dire need to find out a suitable land management practice along with optimum irrigation to resolve soil physical constraints and improve crop productivity. This study examined the combined effects of tillage and irrigation on soil water transmission characteristics, yield and water productivity of wheat and maize in a semi-arid sub-tropical environment of Punjab in northwest India.

Materials and methods

A field study was conducted at research farm of Department of Soil Science, Punjab Agricultural University, Ludhiana (30° 56’ N, 75° 52’ E, 247 m above the mean sea level), Punjab, India. The area is characterized by sub-tropical and semi-arid type of climate with hot and dry summer from April to June followed by hot and humid period during July to September and cold winters from November to January. The average rainfall of the area is 600-700 mm, of which about 80 percent is received during July to September. The soil was non calcareous, non-saline, neutral with medium organic carbon content.

The treatments includes four tillage practices i.e. no-tillage with residue (NT), strip tillage (ST), conventional tillage (CT) and deep tillage (DT). In NT practice, the surface residue was retained and sowing of both wheat and maize was done directly in standing stubbles and loose straw of previous crop, however, in ST, the seedbed is tilled in strips leaving the residue in between undisturbed. The conventional tillage (CT) involved five field operations i.e. two disks, two cultivator followed by one planking operation, whereas in DT the deep ploughing of soil was done up to 45 cm followed by CT. The irrigation treatments include three irrigation water over pan evaporation (IW/PAN-E) ratios, 1.2, 0.9 and 0.6. The experiment was conducted in split plot design with three replications. The tillage treatments were kept in main plots and irrigation in sub plots. The recommended dose of fertilizers as per Punjab Agricultural University package of practices were applied at the rate of 125 kg N ha^{-1} in the form of urea, 62.5 kg P_2O_5 ha^{-1} in the form of single superphosphate and 30 kg K_2O ha^{-1} in the form of muriate of

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potash to both the crops. The wheat and maize crops were sown with the recommended seed rate of 100 and 20 kg ha^{-1}, respectively. Weeds were kept under check with use of recommended herbicides and hand weeding. The manual harvesting of wheat was performed in mid April, while decobbing of harvested maize crop was done mechanically after sun drying and the seed yield was recorded from each plot.

Infiltration was measured, *in-situ* at the end of cropping cycle by double ring infiltrometer method according to Reynolds et al. (2002). Water was filled in both the outer and inner rings and the fall of water levels in the inner ring was recorded at different time intervals till the water intake rate becomes constant. The saturated hydraulic conductivity (K_s) was determined using constant head method (Reynolds et al., 2002). Undisturbed soil cores (8 cm diameter and 7.5 cm length) were collected from 0-7.5 and 7.5-15 cm depths. Samples were saturated in the laboratory by placing on cloth covered perforated disks in 25 cm deep tray containing 5 cm of water. Saturated soil sample along with core was connected with another core and to avoid the water leakage grease was applied in the jointing place on the top of previous core. A thin layer of water was slowly poured on top of the sample by using siphons connected to a constant head device (Mariotte apparatus). The volume of water that percolates through the sample was measured at definite intervals of time. The K_s was calculated using the following equation:

$$K_s = (Q/At) \times \{L/(H+L)\} \quad \text{(Eq.1)}$$

where,
Ks = saturated hydraulic conductivity (cm h^{-1}),
Q = volume of percolate collected (cm^3),
L = length of soil column (cm),
A = cross sectional area of soil column (cm^2),
t = time (h),
H = depth of water above soil (cm).

For determining soil bulk density (ρ_b) the undisturbed soil cores were taken at the end of cropping cycle upto 30 cm depth (0-7.5, 7.5-15, 15-22.5 and 22.5-30 cm) with the help of cylindrical core (7.5 cm height and 8.0 cm diameter) and dried in an oven at 105 °C till the weight of the soil becomes constant. The ratio of dry soil mass (M_s) and internal volume (V_t) of the cylindrical ring is expressed as bulk density (ρ_b) of soil (Mg m^{-3}) (Blake and Hartge, 1986).

$$\text{Bulk density } (\rho_b) = M_s/V_t \quad \text{(Eq.2)}$$

The penetration resistance (PR) was measured by a hand-held digital cone penetrometer (CP40II; Rimik electronics, RFM Australia) at three randomly selected locations within a plot. The soil PR readings were recorded up to 60 cm depth. The measurements were made at the end of the cropping cycle along with simultaneous measurements of soil moisture content.

Soil moisture samples were taken for entire soil profile (0-120 cm soil depth with 15 cm increments) with screw auger. Moisture storage in different layer was computed by multiplying the mass water content depth of a particular layer and bulk density. It was summed up for all the layers to get profile moisture storage in cm. Surface soil (0-15 cm) samples were collected for aggregate size analysis Aggregate status of soil was

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determined by wet sieving method (Yoder, 1936). The air-dried soil peds were passed through 8-mm sieve and were retained on 4-mm sieve. Yoder's wet sieving apparatus, comprising of 4 sieve sets, each having nest of 5 sieves of 12.7 cm diameter and 5 cm height and with hole sizes of 2.0, 1.0, 0.5, 0.25 and 0.1 mm (with mesh numbers of 8, 16, 32, 64 and 150 respectively), were used for this purpose. The samples were evenly distributed over the top sieve of the sieve sets and pre-wetted by capillarity for 10 minutes. The nest of sieves was then allowed to move up and down for 30 minutes. Following this, the sieves were drawn out of water and the oven-dried weight of aggregates retained on each sieve was recorded after drying these in an oven at 105 °C till the constant weight achieved. Mean weight diameter (MWD) and water stable aggregates (WSA) were calculated using the formula:

$$\mathrm{MWD} = \sum_{i=1}^{n} d_i \times w_i \quad \text{(Eq.3)}$$

$$\mathrm{WSA} > 0.25\ \mathrm{mm}(\%) = \frac{\sum_{i=1}^{n} w_i}{\text{weight of sample}} \times 100 \quad \text{(Eq.4)}$$

where, n is number of size fractions (the finest fraction that passes through the finest sieve inclusive), d_i is the mean diameter of each size range, w_i is the weight of aggregates in that size range as a fraction of the total dry weight of the sample analysed. Oxidizable soil organic carbon was estimated using (Walkley and Black, 1934) rapid titration method, using a diphenyl amine indicator.

The plant height of ten randomly selected plants in each plot was measured with the help of meter scale from ground surface to apex of the plant at 70 days after sowing (DAS) in maize and 100 DAS in wheat. The root distribution was measured at 70 DAS in maize and 100 DAS in wheat. The root samples were collected from 0-15, 15-30, 30-45 and 45-60 cm soil layers. For root sampling, the soil cores were taken with the help of core sampler of 5 cm diameter. Samples were taken in between the plant rows. The root-soil cores were then collected and washed in plastic nets. Roots were carefully separated from the soil by washing the nets under water. The washed roots were further cleaned to remove any leftover weed roots, seed and other organic debris. The root length density (RLD) (cm cm^{-3}) was calculated from the total length of roots measured by scanner to the volume of the core. A representative sample of one thousand grains of maize from each plot was counted manually and weighed on a precision balance and expressed in grams. However, a representative sample of one thousand grains of wheat from each plot was counted with the help of automatic seed counter (SLY-C Automatic Seed Counter, Shailron Technology Pvt. Ltd.) and weighed on a precision balance and expressed in grams.

The crop biomass determination was made by taking all the above ground plant parts at time of harvesting. The samples were allowed to dried and then weighed to express in Mg ha^{-1}. The harvested crop produced from each plot was thrashed in case of wheat and decobbed in maize. Grain yields of both the crops were recorded in kg from 24 m^2 area in each plot and finally expressed in Mg ha^{-1}. The water productivity was calculated by dividing the grain yield of corresponding treatments of both the crops with the total water use (irrigation water + rainfall + profile water use) in particular treatment. The data

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collected on various aspects of the investigations were statistically analyzed as prescribed by Cochran and Cox (1967) and adapted by Cheema and Singh (1991) in statistical package CPCS-I. The treatment comparisons were made at 5 per cent level of significance.

Results and discussion

Bulk density (ρ_b) and penetration resistance (PR)

Soil mechanical characteristics including ρ_b and PR affect other soil properties as well as the crop productivity. The data presented in *Figure 1* showed that at subsurface soil depth (15-30 cm), where the chances of occurrence of hard pan were prominent yielded highest ρ_b (Mg cm^{-3}) under CT (1.68) and minimum under in DT (1.52). Similar observations were recorded for other soil depths. The other two tillage systems recorded in between values. The use of heavy machinery and intensive tillage in CT for performing five field operations (twice disc, twice cultivators and one planking operation) can increase soil ρ_b at the ploughing depth (15 to 30 cm). Whereas, in case of DT the soils was shattered up to 45 cm depth, which break down the compaction and reduces ρ_b of soil. The DT experienced an all-time low ρ_b. However, the lower ρ_b in NT plots may be associated with greater soil biological activates, especially earthworms (Lal, 1976; Kahlon et al., 2012a). Jin et al. (2011) also reported that the mean ρ_b at 0-10 and 10-20 cm soil layers under NT practice was 2.1 per cent and 4.7 per cent significantly ($P< 0.05$) lower than under CT. In general with increase in depth there was an increase in ρ_b. The PR determines the soil strength which effect root proliferation. The PR measurements of soil can be used to assess the need for tillage operations, which will help in maintaining effective plant rooting and facilitate good water and nutrient uptake. In general, root tips are unable to penetrate pores narrower than their diameter. They can exert a vertical pressure ranging from 0.7 to 2.5 MPa, depending on crop species. Likewise soil ρ_b, the PR also showed maximum value in CT (2.8 MPa) and minimum in DT (1.9 MPa) at subsurface soil depth (15-30 cm) (*Figure 1*). This indicates the formation of hard pan, which restricts the downward movement of water and root penetration. Since the PR linked with the soil moisture content, its value decreased at lower soil depths. Maximum difference in PR values were observed between DT and CT at subsurface soil depth i.e. 15-30 cm. Kahlon et al. (2012b) reported higher soil PR in CT than NT. Penetrometer values greater than 2 MPa are generally reported to reduce root growth significantly (Atwell, 1993). In well-structured soils or those in which biochannels are preserved (as in non-tilled soils); roots continue to extend at greater penetrometer readings because they can grow in integrated space.

Water transmission characteristics

Soil management practices affect water transmission characteristics to such an extent that they may cause considerable loss to crop and water productivity. Significant differences ($P< 0.05$) in K_s were observed among tillage practices for 0-7.5 and 7.5-15 cm depths (*Figure 2*). The highest K_s (cm h^{-1}) were observed in DT (4.9 and 3.7) and the least under CT (3.8 and 2.2) at 0-7.5 and 7.5-15 cm depths, respectively. The higher K_s under DT may be due to more macro porosity. However, the NT plots showed more K_s than CT and ST. The presence of abundant roots and biochannels are responsible for rapid conduit of water through the soil under NT. The pore continuity maintained due to better aggregate stability and pore geometry also leads to higher K_s under NT. The

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activity and population of soil organisms may also have played an important role in increasing pore continuity. The pores were more continuous under NT plots, probably because of more soil fauna and preceding crop root channels (Singh et al., 1995; Bhattacharyya et al., 2006; Kahlon et al., 2012b). In general, K_s decreased with increase in depth in all tillage practices. Due to more soil disturbance and trafficking the macro pores reduced under CT which leads to lowest K_s.

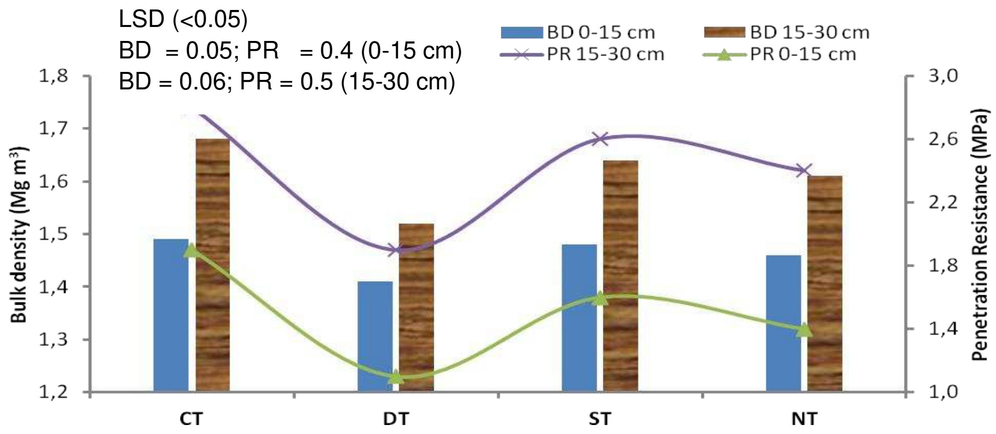

Figure 1. *Effect of tillage practices on soil bulk density and penetration resistance recorded at the end of wheat-maize cropping system (DT = deep tillage; CT = conventional tillage; ST = strip tillage; NT = no-tillage)*

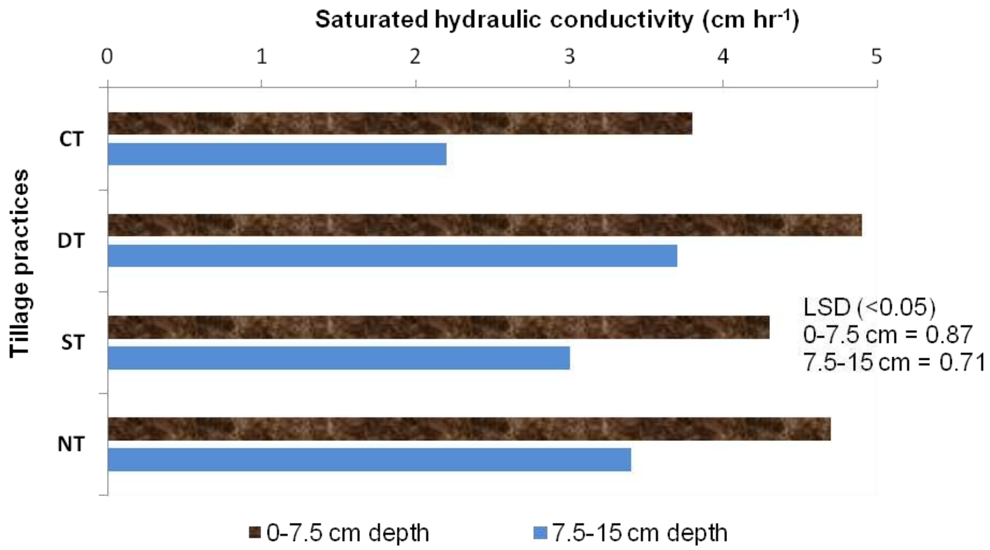

Figure 2. *Saturated hydraulic conductivity as affected by tillage practices at 0-7.5 and 7.5-15 cm soil depth measured in wheat-maize cropping system (DT = deep tillage; CT = conventional tillage; ST = strip tillage; NT = no-tillage)*

Final infiltration rate (IR) was also found to be significantly affected by tillage practices (*Table 1*). Mean highest IR (cm h^{-1}) was observed under DT (3.9), followed by NT (3.5), ST (3.1) and CT (2.7). The higher infiltration recorded under DT attributes to more macro porosity which leads to fast entry of water into the soil profile. The NT still

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recorded more IR than CT and ST. The reason cited for higher IR under NT than CT may be due to higher SOC content, continuity of water conducting pores, biochannels and MWD which led to a better pore size distribution (Azooz et al., 1996; Jabro et al., 2008). In pattern of IR the maximum cumulative infiltration (CI) was observed under DT (30.6 cm) followed by NT (24.3 cm), ST (21.7 cm) and CT (18.2 cm) (*Table 1*). The higher IR observed under DT reflect the breakdown of hard pan which restricts downward entry of water into the soil. As concluded by Shaver et al. (2002), more number of macropore channels in DT aided in better water transmission characteristics as compared to other tillage treatments. This may also be due to less stable aggregates in the CT practice which upon intense rainfall event clogs the soil pores through slaking of aggregates leading to decrease in water transmission through the soil as also reported by Mbagwu and Auerswald (1999). Further, less soil disturbance in the NT also kept pore structure continuous which aids greater water transmission through the soil. Another reason cited by the researchers to support conservation tillage is that the crop residues left on the soil surface limit evaporation, soil sealing and crusting and thereby increase soil infiltration (Gangwar et al., 2006). Water transmission through the soil profile also depends on the antecedent water content, aggregation and the presence of macropore channels. Least value was observed under CT might be due to relatively smaller pore heterogeneity, discontinuity of pores and less stable aggregates. Maximum water holding capacity (%) and soil moisture storage (cm) was observed in NT (41.8 and 9.6), respectively. Whereas, the minimum values of both were recorded under CT (*Table 1*).

Table 1. *Soil physical characteristics under different tillage practices in wheat- maize system*

Tillage practices	MWD (mm)	WSA (>0.25 mm, %)	IR (cm h^{-1})	CI (cm)	WHC (%)	SMS (cm)
CT	0.39	28.9	2.7	18.2	39.8	8.7
ST	0.48	36.1	3.1	21.7	41.4	9.3
NT	0.58	41.6	3.5	24.3	41.8	9.6
DT	0.36	27.4	3.9	30.6	40.3	8.8
Mean	0.45	33.5	3.3	23.7	40.8	9.1
LSD (<0.05)	0.19	2.8	0.5	4.2	1.6	0.6

CT: conventional tillage; ST: strip tillage; NT: no-tillage; DT: deep tillage; MWD: mean weight diameter; WSA: water stable aggregates; IR: infiltration rate; CI; cumulative infiltration; WHC: water holding capacity; SMS: soil moisture storage

Mean weight diameter (MWD), water stable aggregates (WSA > 0.25 mm) and soil organic carbon

Tillage practices experienced a significant effect on soil aggregation as observed from the data for both the indicators i.e. MWD and WSA (>0.25 mm) (*Table 1*). Among tillage practices, the mean highest MWD (mm) of 0.58 was found under NT followed by ST (0.48), CT (0.39) and least under DT (0.36). Mathematical representation foretells that the mean highest WSA (> 0.25 mm, %) of 41.6 was observed in NT followed by ST (36.1), CT (28.9) and DT (27.4). The DT showed significantly lower values of MWD and WSA in comparison to other treatments. The higher soil aggregation observed in NT attributes to the non disturbed soil conditions and retention of crop residue on soil surface, which helps the aggregates to break from direct raindrop

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impacts as well as with addition of organic matter in the soil. Different aggregate size fractions (per cent) as affected by tillage practices are presented in *Figure 3*. The data on aggregate size distribution indicate that greater proportion of smaller particles with size < 0.25 mm was found in DT as compared with other tillage treatments. However, the proportion of larger size aggregates (> 1.0 mm) follows the order NT > ST > CT > DT (*Figure 3*). It is well established fact that soil aggregation correlated significantly with the organic carbon content of soil. The same was reported in present study, where, the SOC content was more i.e. under NT practice the bigger size aggregates were also recorded in that treatment. The NT recorded 28 % more SOC than DT (*Figure 4*).

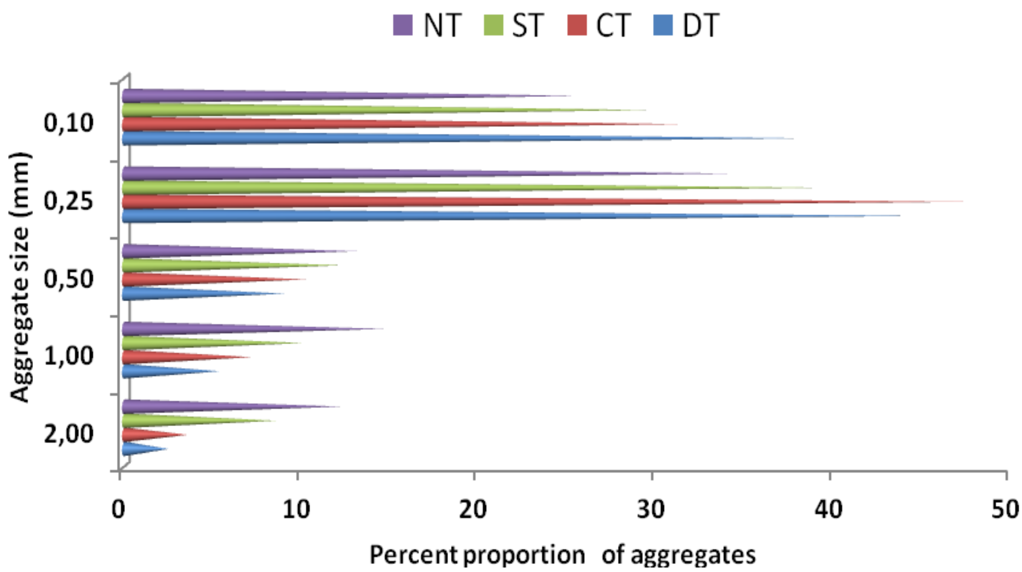

Figure 3. *Percent proportion of aggregate size fractions under different tillage practices in wheat-maize cropping system (DT = deep tillage; CT = conventional tillage; ST = strip tillage; NT = no-tillage)*

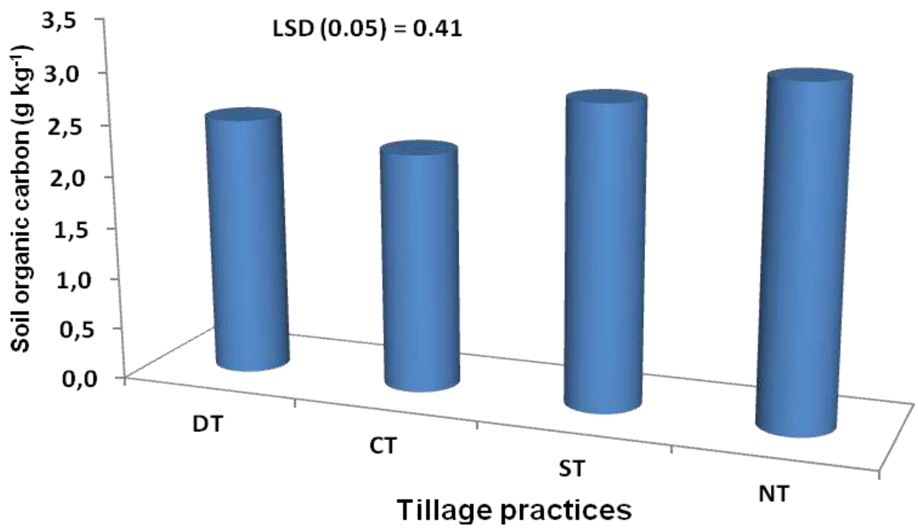

Figure 4. *Soil organic carbon under different tillage practices in wheat-maize system (DT = deep tillage; CT = conventional tillage; ST = strip tillage; NT = no-tillage)*

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Root length density (RLD)

In general, the increase in subsoil compaction resulted in reduced rooting system. According to the data presented in *Table 2*, it was observed that due to high soil ρ_b and PR in subsurface soil layer (15-30 cm), CT recorded the least RLD while its maximum value was recorded in DT. However, due to more availability of soil moisture and nutrients in surface layer (0-15 cm) the NT had more RLD than CT and ST. Alternatively, the main reason for higher RLD in DT is the removal of compact hard pan below surface soil layer which otherwise restrict the root proliferation to lower soil depths. Similar observations were recorded for the wheat crop (*Table 2*). The higher root concentration observed in NT attributes to formation of bio channels. However, under CT the presence of plow pan does not allow easy root penetration. Arora et al. (1991) reported that increased tillage reduces soil strength in tilled zone which results in better growth of maize as strength is negatively correlated with root growth. Kumar (2005) studied the effect of tillage on root development of maize and reported that depth of roots at harvest was maximum under DT as compared to CT.

Table 2. *Root length density (cm cm^{-3}) under different tillage practices in wheat- maize system at different soil depths*

Tillage practices	Wheat				Maize			
	Soil depth (cm)							
	0-15	15-30	30-45	45-60	0-15	15-30	30-45	45-60
CT	0.83	0.48	0.22	0.16	1.14	0.62	0.41	0.18
ST	0.78	0.56	0.27	0.21	1.05	0.74	0.47	0.22
NT	1.04	0.59	0.38	0.23	1.23	0.97	0.51	0.25
DT	1.12	0.73	0.42	0.27	1.66	1.11	0.65	0.35
Mean	0.94	0.59	0.32	0.22	1.27	0.86	0.51	0.25
LSD (<0.05)	0.11	0.09	0.06	0.05	0.32	0.18	0.16	0.07

CT: conventional tillage; ST: strip tillage; NT: no-tillage; DT: deep tillage

Plant parameters and productivity of maize and wheat

The data pertaining to tillage effects on plant parameters and productivity of maize and wheat is presented in *Table 3*. It depicts that DT outperformed the other tillage treatments with respect to plant height, thousand grain weight and crop biomass for both the crops. The highest maize grain yield (Mg ha^{-1}) was recorded in DT (6.4) followed by NT (6.3), ST (5.9) and CT (5.5). The same yield pattern was observed for wheat crop. The water productivity (kg ha^{-1} mm^{-1}) was also observed to be maximum in DT (8.8) and least in CT (7.2) for maize crop. The WP of wheat followed the similar trend. The irrigation regimes also significantly affect the plant parameters and crop productivity of both the crops (*Table 4*). Maximum maize grain yield (Mg ha^{-1}) was recorded at irrigation regime $I_{0.9}$ (6.5) followed by $I_{1.2}$ (6.2) and least under $I_{0.6}$ (6.1). Irrigation regimes also showed significant effect on water productivity (WP). The mean highest water productivity (kg ha^{-1} mm^{-1}) was found under $I_{0.9}$ (8.7) and the lowest under $I_{0.6}$ (7.8) for maize. The water productivity decreased with increase in IW/PAN-E ratio (0.6 to 1.2). Memon et al. 2013 also reported higher maize grain yield in DT than NT and CT treatments. The increase in yield with increase in number of irrigations is because of

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more availability of water for plant physiological processes. Mojid et al. (2009) also reported decrease in water productivity with increase in irrigation level.

Table 3. *Plant height, thousand grain weight, crop biomass, crop yield and water productivity of wheat and maize under different tillage practices*

Tillage practices	Plant height (cm)		Thousand grain weight (g)		Crop biomass (Mg ha^{-1})		Crop yield (Mg ha^{-1})		Water productivity (kg ha^{-1} mm^{-1})	
	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize
CT	0.84	2.21	35.8	253.7	12.8	14.9	3.8	5.5	18.7	7.2
ST	0.92	2.42	37.6	267.6	13.2	15.2	4.0	5.9	19.4	7.6
NT	0.88	2.57	39.5	287.5	14.1	16.3	4.1	6.3	20.6	8.1
DT	1.12	2.62	42.3	301.4	14.6	17.6	4.3	6.4	22.2	8.8
Mean	0.94	2.46	38.8	277.6	13.7	16.0	4.0	6.0	20.2	7.9
LSD (<0.05)	0.08	0.18	3.6	16.2	0.8	1.4	0.3	0.51	1.7	0.8

CT: conventional tillage; ST: strip tillage; NT: no-tillage; DT: deep tillage

Table 4. *Plant height, thousand grain weight, crop biomass, crop yield and water productivity of wheat and maize under different irrigation regimes*

Irrigation regimes	Plant height (cm)		Thousand grain weight (g)		Crop biomass (Mg ha^{-1})		Crop yield (Mg ha^{-1})		Water productivity (kg ha^{-1} mm^{-1})	
	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize	Wheat	Maize
I1	1.02	2.49	41.5	280.6	14.4	16.2	4.3	6.2	21.3	8.2
I2	0.95	2.58	37.6	282.6	13.5	16.6	4.0	6.5	20.2	8.7
I3	0.89	2.30	35.9	269.3	13.1	15.7	3.8	6.1	19.4	7.8
Mean	0.95	2.46	38.3	277.5	13.7	16.1	4.0	6.3	20.3	8.2
LSD (<0.05)	0.09	0.21	2.2	7.4	0.7	0.6	0.3	0.3	0.9	0.4

I1: IW/ PAN-E ratio 1.2; I2: IW/ PAN-E ratio 0.9; I3: IW/ PAN-E ratio 0.6 (for both wheat and maize)

It is concluded that under compaction or hard pan formation at subsurface soil layer the deep tillage (DT) is most appropriate practice while under water stress situation the no-tillage with residue retention (NT) is viable option for achieving higher crop and water productivity in maize-wheat system in northwest India.

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MICROBIAL ACTIVITY AND COMMUNITY DIVERSITY IN TOBACCO RHIZOSPHERIC SOIL AFFECTED BY DIFFERENT PRE-CROPS

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Abstract. In this study, we used both culture-dependent physiological profiling and culture-independent DNA-based approaches to characterize the bacterial communities of tobacco rhizospheric soils affected by different pre-crops (soybean, maize and tobacco). Using Biology-Eco plates, we found that the bacterial metabolic activity in soybean-tobacco and maize-tobacco rotation soils were higher than in tobacco monocropping soil. Across all soil samples, bacterial communities were dominated by *Proteobacteria*, *Acidobacteria* and *Actinobacteria* at the phylum level. However, the diversity and composition of the bacterial communities varied significantly between tobacco rotations and monocropping soil. The estimated bacterial diversity (Shannon diversity index) was higher in the maize-tobacco and soybean-tobacco soils than in tobacco monocropping soil. The populations of *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Bacteroidetes* were found in variable proportions, depending on the different pre-crops. The highest percentages of *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were found in soybean-tobacco soil, whereas *Acidobacteria* occurred at higher percentages in tobacco monocropping soil. Collectively, crop rotation influenced soil biodiversity by change of composition and abundance of individual species, and soils under cereal-tobacco rations had higher bacterial activity and diversity than soils under tobacco monocropping.
Keywords: *soybean*, *maize*, *monocropping*, *rotation*, *bacterial community structure*, *bacterial community function*

Introduction

Microorganisms are ubiquitous in the environment and play an essential role in the biogeochemical cycles that sustain all life on Earth (Zarraonaindia et al., 2013; Su et al., 2012). Soil microbial communities are responsible for soil structure maintenance,

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organic matter decomposition, nitrogen fixation, the breakdown of toxic compounds, and inorganic compound transformation (Dick, 1992; Nannipieri et al., 2003). Previous studies have shown that the community structure, abundance and activity of soil microorganisms can be affected by various physical disturbances in the soil (Dick, 1992; Lienhard et al., 2013). Thus, land-use management practices, including crop rotation and tillage (Quadros et al., 2012), fertilizer regime (Qiu et al., 2012), irrigation (Qiu et al., 2012), and continuous cropping (Li et al., 2010) are logical case studies for soil microbial diversity studies.

Tobacco (*Nicotiana tabacum* L.) is among the most important economic crops in the world, and one million acres of tobacco are planted each year in China (Hecht et al., 1984). Tobacco quality is determined by genetic factors, environmental factors and cultivation measures. Cereal-tobacco rotation is the dominant practice in the northeast of China. Soybean (*Glycine max* L.) and maize (*Zea mays* L.) are the major cereal crops grown most often in rotation with tobacco. However, research on the relative effects of the preceding crops on the soil quality of subsequent tobacco in cereal-tobacco rotation systems is rare. It is well known that rotation improves soil quality, including soil biota structure, organic matter content and moisture retention capacity (Ponge et al., 2013). Soils under crop rotation with a high input and diversity of organic materials contain high microbial biomass content and enzyme activity compared with monoculture soils (Trasar-Cepeda et al., 2008). Many studies have found that crop sequence plays a major role in soil C retention (Morari et al., 2006; Varvel, 2008; Wright and Hons, 2005). In particular, the effects of preceding crops on subsequent crops can relate to residual nutrients and water as well as to disease control (Kirkegaard et al., 2008). The decomposition rate of plant debris was found to be mainly governed by the C/N ratio. There is growing evidence that the variety of pre-crops can become factors in the microbial population and enzymes in the soils for subsequent crops.

In this study, two different techniques (Biolog-Eco and 454 pyrosequencing) for estimating the soil bacterial community were adopted. To build a sustainable crop-tobacco rotation sequence, the objective was to investigate the bacterial community structure and function of subsequent tobacco in soils in response to three preceding crops (soybean, maize and tobacco) and to observe differences among bacterial communities.

Materials and Methods

Field plots and sample collection

Soil sampling was performed in September 2011, at Tobacco Research Institute of Mudanjiang, Ningan city of Heilongjiang province (latitude, 44°85′N; longitude, 129°60′E, Northeast China). The average annual temperature in the region is 4°C. The mean annual precipitation is 427.50 mm, and the evaporation is 1635 mm per year. The soil is classified as a river silt soil. Selected soil properties were as follows: pH 6.85; organic matter 27.70 $g \cdot kg^{-1}$; total nitrogen 1.90 $g \cdot kg^{-1}$; total phosphorus 1.62 $g \cdot kg^{-1}$; total potassium 1.61 $g \cdot kg^{-1}$; available nitrogen 86.50 $mg \cdot kg^{-1}$; available phosphorus 36.40 $mg \cdot kg^{-1}$; available potassium 300.00 $mg \cdot kg^{-1}$.

The experimental design consisted of three blocks. Each block was divided into three plots representing the three plantation systems. Each plot comprised 8 rows that were 6.00 m long and 8.80 m wide; thus, each was 52.80 m^2 in size. Treatments levels included (1) tobacco monocropping, (2) soybean-tobacco rotation and (3) maize-tobacco rotation. The cultivars of tobacco, soybean and maize were Longjiang 911, Heinong 34 and Kenfeng 1, respectively. Soil samples were collected from all

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plots, three individual rhizosphere soils randomly collected for each treatment in a plot. Tobacco rhizosphere soils, which adhered to the roots (Nazih et al., 2001), were collected by shaking the soil off the roots. After removal of the vegetation, roots, and stone (>2 mm), samples were placed into sterile centrifuge tubes under ice and transported to the laboratory within 24 hours. Finally, samples were stored at 4°C prior to microbial functional (Biolog™ ECO technic) and structural (454 pyrosequencing) analyses.

Substrate utilization patterns and data analysis

Substrate utilization patterns were measured using Biolog™ ECO plates (BIOLOG, Inc.). A 10^{-1} microbial suspension was prepared by suspending 10 g of fresh soil in 100 mL 0.85% NaCl solution. The slurry was processed with a Vortex mixer for 1 min at maximum speed and centrifuged for 10 min at 500×g (Rutgers et al. 2006). Tenfold serial dilutions were performed, and 150 μL of the 10^{-3} dilutions were pipetted into microplates using an 8-channel micropipette. Microplates were incubated at 28°C for 216 h. The color development at OD_{578} nm was read for each well at 24-h intervals. Negative values were set to zero. The average well color development (AWCD) value of the Biolog data was calculated for each sample at each time point by dividing the sum of the optical density data by 31 (number of substrates), as described by Garland (1996).

The 96-h data were used to measure the functional and species diversity of the soil microbial community, and the following parameters were calculated using the equations below:

Shannon-Wiener index $$H=-\sum Pi\times\ln(Pi)$$ (Eq.1)

Simpson index $$D=1-\sum(Pi)^2$$ (Eq.2)

McIntosh index $$U=\sqrt{\left(\quad Ni^2\right)}$$ (Eq.3)

where Ni is the relative OD in each carbon source well, and Pi is calculated by subtracting the control from the absorbance of each substrate and then by dividing this value by the total color change recorded for all 31 substrates.

DNA extraction, amplification of 16S rRNA genes, and pyrosequencing

Total microbial community DNA was isolated from 0.25 g of soil per sample using soil DNA extraction kit (Omega Bio-Tek, Atlanta, USA). The extracted DNA was examined following electrophoresis in 1% agarose gel, and the DNA was normalized to the same concentration prior to amplification. A ~455 bp region of the 16S rRNA gene covering the V1-V3 region was selected to construct the community library through tag pyrosequencing. The V1-V3 region was amplified using the universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') containing the A and B sequencing adaptors (454 Life Sciences). The PCR mixture (final volume, 50 μL) contained 5 μM of each primer, ~5 ng of template DNA, 5×FastPfu PCR buffer, and 2.5 U of FastPfu DNA Polymerase (MBI, Fermentas, USA). The amplification conditions consisted of an

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initial denaturation at 95°C for 2 min and 25 cycles of denaturation at 95°C at 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension period at 72°C for 5 min. During amplification, negative control reactions lacking template DNA were also performed to check for experimental contamination. The amplicons were then purified once by gel electrophoresis/isolation and twice more using the Wizard SV Geland PCR Clean-Up System (Promega, Madison, Wisconsin, USA). Next, 454 pyrosequencing was conducted on a Roche massively parallel 454 GS-FlX sequencer according to standard protocols.

454 Pyrosequencing and data analysis

Pyrosequencing flowgrams were converted to sequence reads using the MOTHUR software (http://www.mothur.org) and analyzed. The acquired sequences were filtered by evaluating data quality and removing primers and barcodes. Sequences were filtered by the following methods: (1) selecting sequences that contained the barcode and forward primer and eliminating sequences with even a single base pair; (2) removing sequences shorter than 150 bp, with ambiguous base pairs, or with more than two wrong matches in the primer; and (3) eliminating barcodes and forward primers. After filtering, the effective sequences were clustered into operational taxonomic units (OTUs) based on phylum, class, order, family, genus and species levels using MOTHUR.

Data analysis

To detect OTUs, optimized sequences were reduced to 300 bp in length and compared using silva108. Rarefaction curves based on an identified OTU, the species richness estimator Chao 1, were generated for each sample using MOTHUR. The taxonomic assignment of sequences was performed using the Ribosomal Database Project (RDP) classifier (minimum confidence of 80%). After phylogenetic allocation of the sequences to the phylum and genus levels, the relative abundance of a given phylogenetic group was set to the number of sequences per sample. Hierarchical cluster (Heatmap) analyses were generated in MOTHUR using the gplots package of R. We used SPSS for windows (version 19) to test for significance (P<0.05) between treatments of relative abundances, Alpha diversity and richness of bacterial communities were analyzed using Duncan post-hoc test at 95% confidence level. The Duncan test method was conducted for multiple comparisons to assess the significance level of substrate utilization among treatments.

Results

Community level physiological profile

As expected, the average well color development (AWCD) increased with incubation period (*Fig. 1*). The soybean-tobacco rotation consistently exhibited the highest AWCD at all periods, followed by maize-tobacco rotation. There was no significant difference in soil bacterial functional diversity between the soybean-tobacco and maize-tobacco treatments. The tobacco monocropping treatment constantly had the lowest AWCD throughout all period. The soil bacterial functional diversity index (H, D and S) was significantly affected by the pre-crop treatment (*Table 1*). Among rotation treatments, there was no significant difference between the soybean-tobacco and maize-tobacco treatments.

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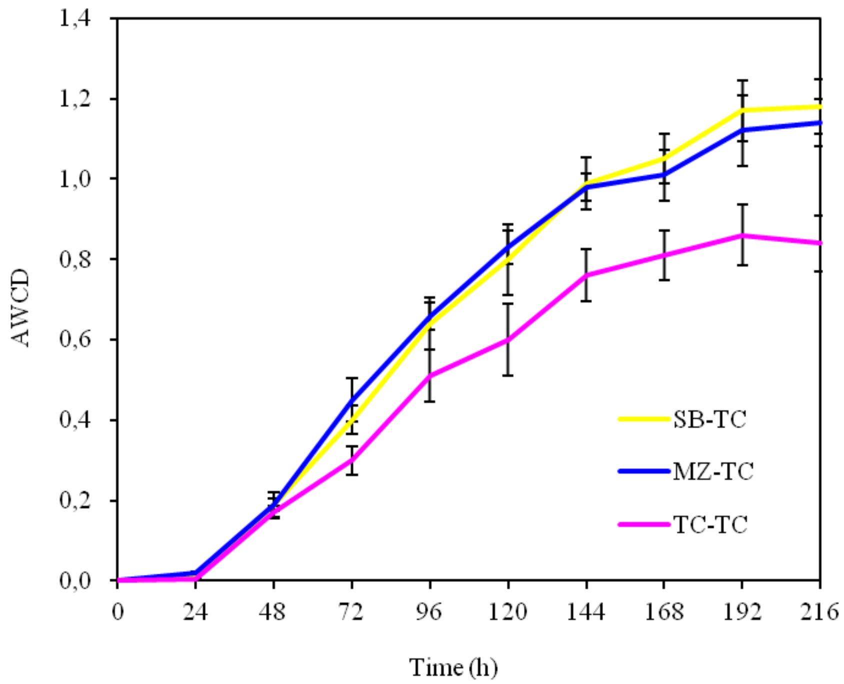

Figure 1. *Average well color development (AWCD) obtained by Biolog-Eco Plate™ incubation of all treatments. SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

Table 1. *Bacterial diversity indices of soils based on community level physiological profile. Treatment: SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping. Different letters indicate significant difference at P<0.05*

Treatment	Shannon-Wiener index(*H*)	Simpson-diversity index(*D*)	Substrate richness (*S*)	McIntosh index(*U*)
SB-TC	1.31±0.01a	0.94±0.00 a	23.33±0.58 a	4.71±0.57 a
MZ-TC	1.32±0.05 a	0.94±0.01 a	24.33±1.53 a	4.64±1.18 a
TC-TC	1.24±0.03 b	0.92±0.01 b	21.33±0.58 b	4.97±0.62 a

Principal component analysis of bacterial community

Microbial consumption of polymers, amino acids, amines/amides and miscellaneous compounds were significantly higher in soybean-tobacco rotation soils compared to that found in tobacco monocropping soil (*Fig. 2*). On the contrary, the consumption of carbohydrates was lower in soybean-tobacco soils than in tobacco monocropping soil. However, similarly to soybean-tobacco rotation treatment, soil microbial communities of maize-tobacco rotation treatment showed large polymers utilization.

The principal component analysis (PCA) of bacterial substrate utilization patterns (*Fig. 3*) explained 79.42% of the total variance, with the first principle component having the greatest power of separation (70.21%). The PCA score plot revealed that the soybean-tobacco and maize-tobacco rotation treatments were clustered together and separated from the tobacco monocropping treatment.

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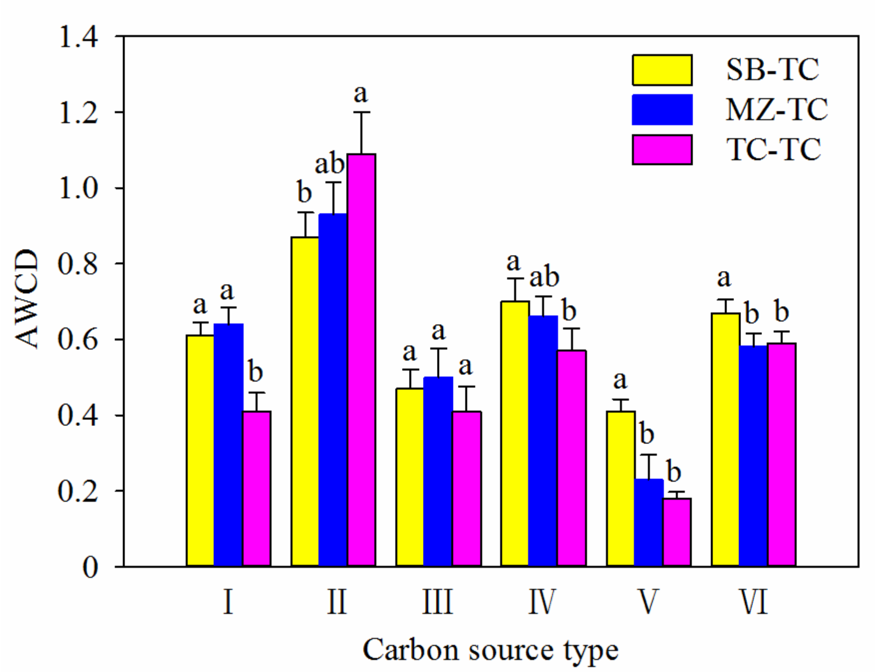

Figure 2. *Relative use efficiency of soil microbial community on different carbon sources. I :Polymers; II: Carbohydrate; III: Carboxylic acids; IV: Amino acids; V: Amines/amides; VI: Miscellaneous. SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping. Different letters indicate significant difference at P<0.05*

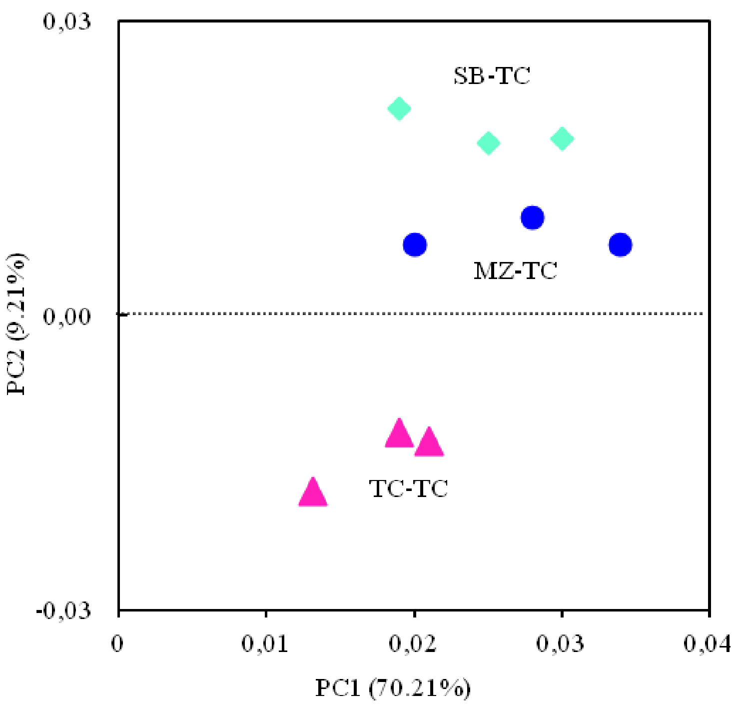

Figure 3. *Principal component analysis (PCA) of the use on different carbon sources for soil microbial community. SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

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Bacterial community analysis using pyrosequencing

In this study, we assessed and compared the composition of soil bacterial communities derived from the soybean-tobacco, maize-tobacco and tobacco monocropping libraries through the pyrosequencing-based analysis of 16S rRNA gene sequences. Pyrosequencing analysis of the V1-V3 region of the 16S rRNA genes resulted in 57,084 high-quality sequences reads with a read length of ≥300 bp across all samples. The average read length was 434 bp. The read numbers were uneven per sample, ranging from 18,343 to 19,911, with an average of 19,028 (*Table 2*). All samples were randomly reduced to the same size using MOTHUR based on the sample with the smallest number of reads.

Table 2. *Number of 16S rRNA gene sequences derived from three libraries. Treatment: SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

Treatment	No. obtained sequences ≥400 bp	Bases (bp)	Average length (bp)
SB-TC	18,343	5,934,166	426.2
MZ-TC	19,911	6,243,854	436.3
TC-TC	18,830	6,091,675	439.7

Bacterial alpha-diversity

To determine rarefaction curves and other measures of diversity, OTUs (operational taxonomic units) were identified at 3% genetic distance. The rarefaction curves indicated consistent differences among the three libraries (*Fig. 4*). At 3% genetic distance, the rarefaction curves suggested that the sequencing effort was not large enough to capture the complete diversity of these communities, as the curves did not reach the asymptote with increasing sample size. The same conclusion was observed in terms of coverage (*Table 3*). The coverage from the three libraries was below 95%, indicating that the sequencing reads were not sufficient for this analysis.

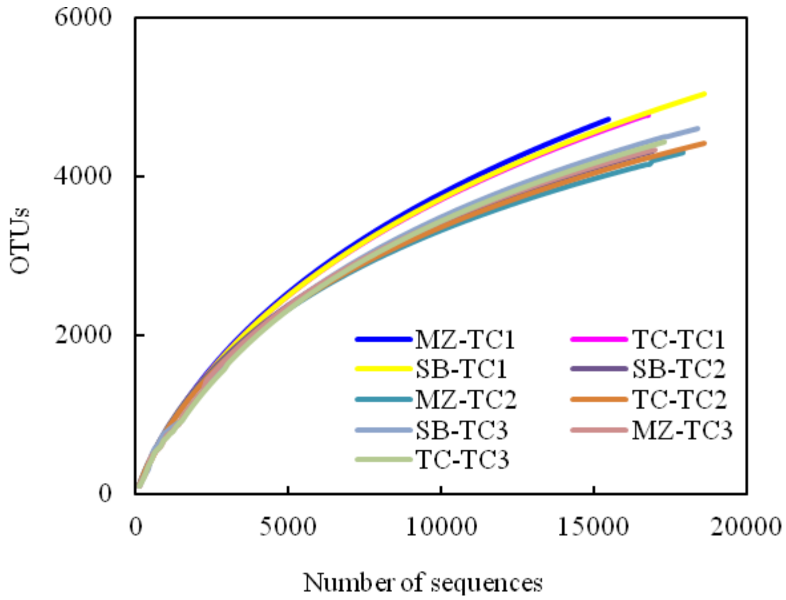

Figure 4. *Rarefaction curves indicating the observed number of OTUs at genetic distances of 3% in all libraries. SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

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Table 3. *Sequencing information and diversity estimated for three samples obtained by 454 pyrosequencing. Treatment: SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

Treatment	3% Genetic distance			
	Ace	Chao1	Shannon	Coverage (%)
SB-TC	8,806	7,221	7.78	86.6
MZ-TC	9,812	7,892	7.91	86.3
TC-TC	7,589	7,563	7.86	86.9

Comparing the mean Shannon diversity index of the three libraries revealed that the highest bacterial diversity at the analyzed genetic distances was found in the maize-tobacco soil, followed by the tobacco monocropping and soybean-tobacco soils. The Chao1 index varied among the three libraries, although not significantly. In addition, the Ace index increased significantly in tobacco rotations soils compared to the tobacco monocropping soil.

Distribution of bacterial composition at the phylum level

The 57,084 classifiable sequences were affiliated with 15 bacterial phyla (*Fig. 5*).The dominant phyla across all samples were *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes*, and *Bacteroidetes*, representing 28.10, 18.90, 17.50, 11.00, 7.59, and 5.47%, respectively, of all sequences that were classified below the domain level. These dominant bacterial phyla were found in all samples (*Fig. 6*). Other sequences belonged to *Nitrospirae*, *Armatimonaddetes*, *Firmicutes*, *Fibrobacteres*, *Chlorobi*, *Verrucomicrobia* and other bacteria, and they were always found in very low proportions (<5%).

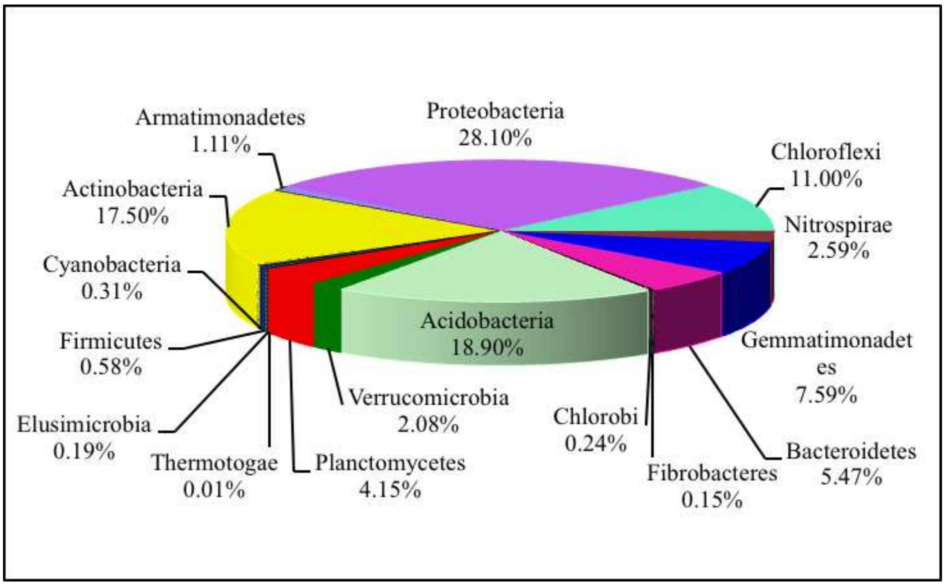

Figure 5. *Proportional distribution of different phyla.*

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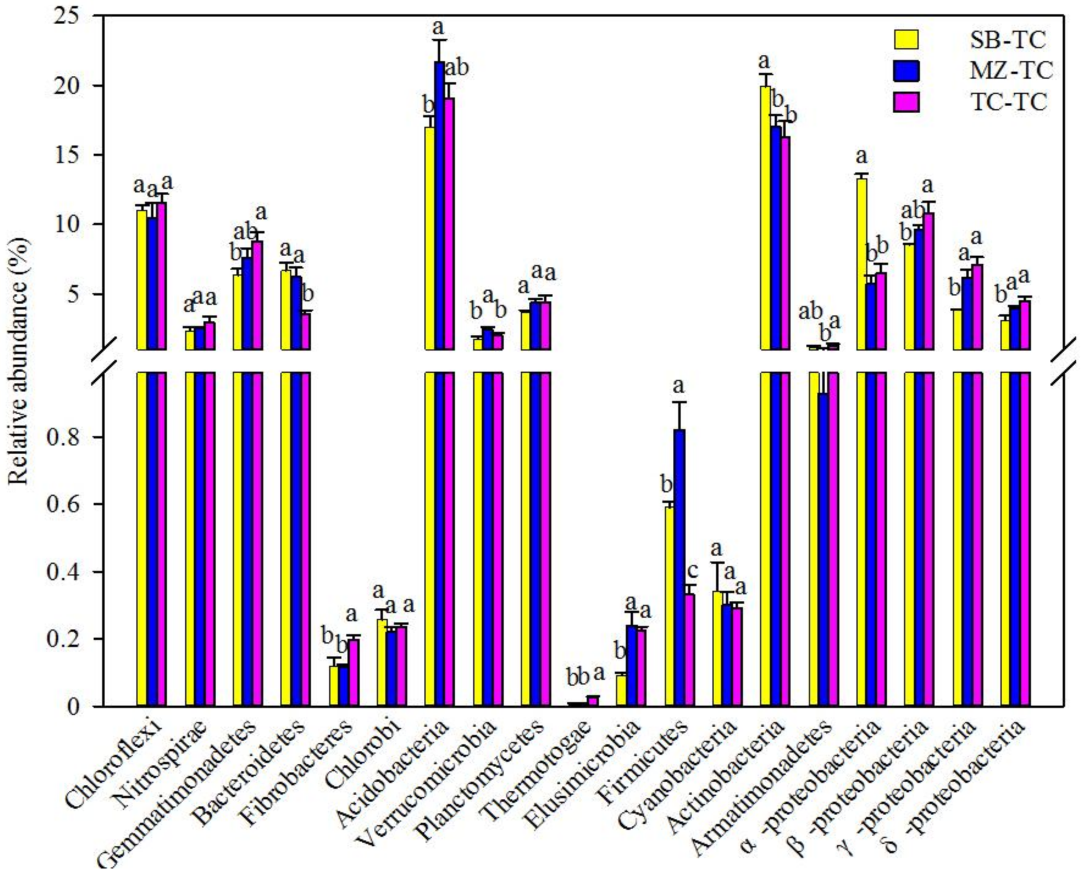

Figure 6. *Relative abundance of bacterial phyla and classes of Proteobacteria for each soil library. SB-TC, soybean-tobacco; MZ-TC, maize-tobacco; and TC-TC, tobacco monocropping*

The bacterial composition at the phylum level differed between the rotations (soybean-tobacco and maize-tobacco) and the monocropping system. The major phyla were shared by all samples, but in different relative abundances (*Fig. 6*). In the maize-tobacco and tobacco monocropping soils, *Proteobacteria* and *Acidobacteria* were the most abundant phyla, representing 26.58%, 28.02% and 20.63%, 19.05%, respectively. However, the soybean-tobacco rotation systems were dominated by the phyla *Proteobacteria* and *Actinobacteria*, representing 29.20% and 19.91%, respectively.

Among the *Proteobacteria*, *α*-, *β*-, *γ*-and *δ-proteobacteria* were found in all soil libraries (*Fig. 6*). In the maize-tobacco and tobacco monocropping systems, *β-proteobacteria* showed the highest relative abundance (9.74% and 10.48%), followed by *γ-proteobacteria* (6.96% and 6.89%), *α-proteobacteria* (5.82% and 6.26%) and *δ-proteobacteria* (4.06% and 4.39%). The insignificant differences between the maize-tobacco and tobacco monocropping soils indicated that maize as a pre-crop did not significantly impact *Proteobacteria* in subsequent tobacco soil (P>0.05). Compared with the tobacco monocropping system, *Proteobacteria* showed significant differences in the soybean-tobacco rotation soil (P<0.05). The relative abundances of *Proteobacterial* sequences were 13.57% for *α-proteobacteria*, 8.67% for *β-proteobacteria*, 3.92% for *γ-proteobacteria* and 3.04% for *δ-proteobacteria*.

Considering the bacterial distribution in tobacco rotations and monocropping systems,

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variations in relative abundances were also observed for the phyla *Acidobacteria*, *Actinobacteria*, *Gemmatimonadetes* and *Bacteroidetes* (*Fig. 6*). The *Acidobacteria* showed relative abundances of 19.05% in tobacco monocropping soil, 16.98% in the soybean-tobacco rotation soil, and 20.63% in the maize-tobacco rotation soil. A statistical comparison across soil libraries revealed that the relative abundances of *Bacteroidetes* in soybean-tobacco and maize-tobacco rotation soils, amounting to 6.62% and 6.23%, respectively, were significantly higher than in tobacco monocropping soil (3.56%)($P<0.05$). In contrast, the relative abundance of *Gemmatimonadetes* revealed a significant decrease in the soybean-tobacco (6.33%) rotation soils compared to the tobacco monocropping soil (8.80%)($P<0.05$). Among the *Actinobacteria*, we observed no significant differences between the soybean-tobacco (19.91%) and maize-tobacco (17.02%) soils ($P>0.05$).

Distribution of bacterial composition at the genus level

Hierarchically clustered heatmap analysis based on 100 predominant bacterial communities at the genus level was used to identify the different compositions of these soil libraries (*Fig. 7*). Variations at the genus level were observed between soil from tobacco rotations and tobacco monocropping soils. Overall, among the *Proteobacteria*, the genera *Rhodoplanes* (0.41%), *Phenylobacterium* (0.53%) and *Sphingobium* (0.86%) revealed a significant increase in soybean-tobacco rotation soil compared to the corresponding genera in the tobacco monocropping soil (0.13%, 0.23% and 0.18%, respectively). In contrast, the relative abundances of *Nitrosomonadaceae* (1.90% and 2.83%), *Sorangium* (0.22% and 0.30%), *Lysobacter* (0.4% and 0.33%) and *Arenimonas* (0.12% and 0.25%) were lower in soybean-tobacco and maize-tobacco soils than in tobacco monocropping soil (3.29%, 0.36%, 0.60% and 0.32%, respectively). The phylum *Actinobacteria*, which contains the genera *Solirubrobacter*, *Aeromicrobium* and *Microbacterium*, showed significant abundance differences between tobacco rotations and tobacco monocropping soils. *Aeromicrobium* showed a higher relative abundance in the soybean-tobacco soil, and *Solirubrobacter* was present at lower relative abundances in the soybean-tobacco and maize-tobacco soils compared with the tobacco monocropping soil. The comparison of the relative abundances of *Solirubrobacter* revealed no significant difference between the soybean-tobacco and maize-tobacco soils. Within the *Bacteroidetes* and *Acidobacteria*, representatives of the genera *Adhaeribacter* and *Candidatus_Chloracidobacterium* were variational in the three libraries. Their relative abundances were significantly higher in the soybean-tobacco and maize-tobacco soils than in tobacco monocropping soil. Other genera, *Bacillaceae_Bacillus* (affiliated with *Firmicutes*), *Zavarzinella* (affiliated with *Planctomycetes*) and *Anaerolineaceae* (affiliated with *Chloroflexi*), showed higher relative abundances in the maize-tobacco soil compared to tobacco monocropping soil.

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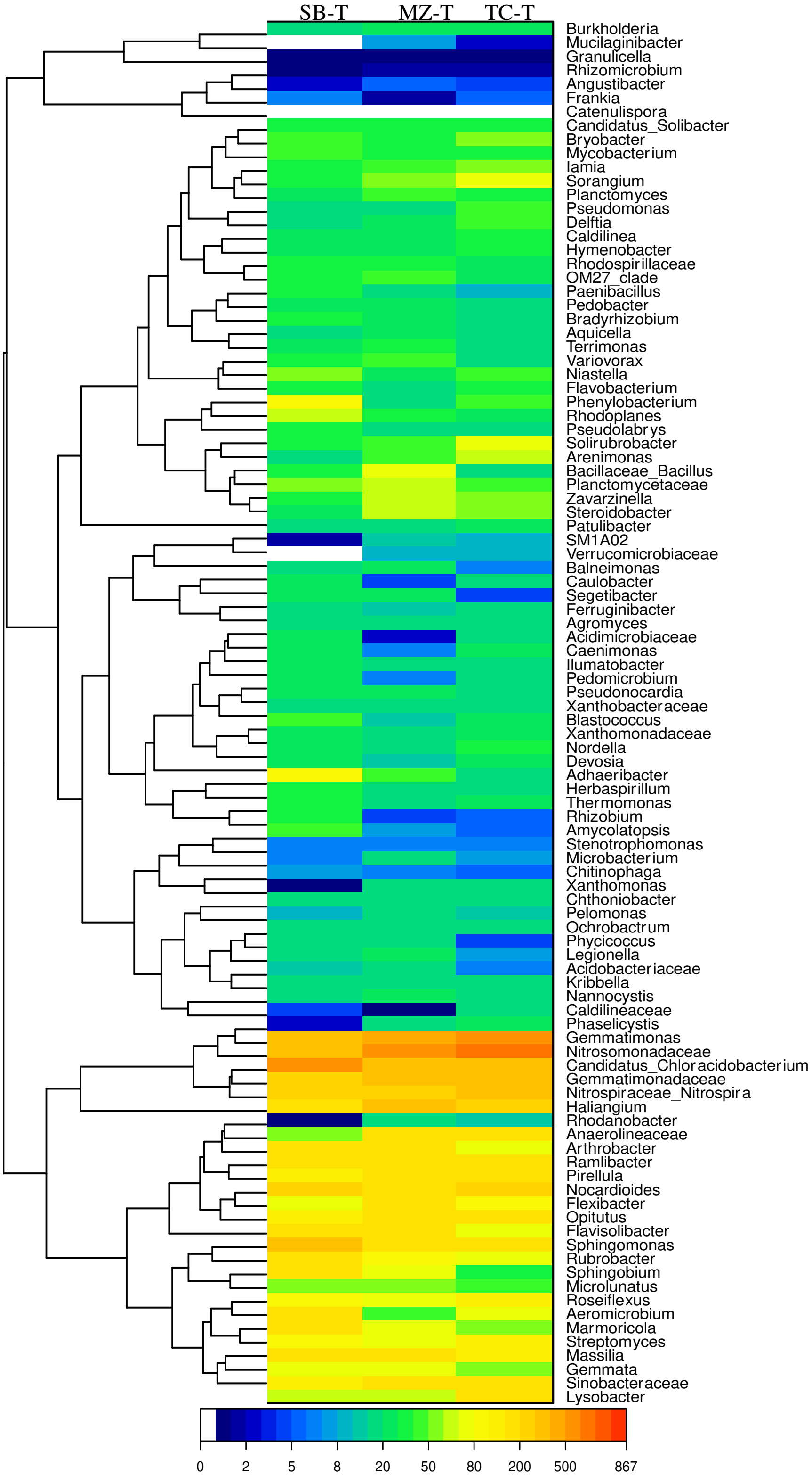

Figure 7. *Hierarchical cluster analysis of 100 predominant bacterial communities among the three samples. The Y-axis is the clustering of the most abundant OTUs (5% distance) in reads. The OTUs were ordered by genus. Sample communities were clustered based on complete linkage method. The color intensity of scale indicates relative abundance of each OTU read. Relative abundance was defined as the number of sequences affiliated with that OTU divided by the total number of sequences per sample*

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Discussion

The residue of the preceding crops has been found to strongly influence soil characteristics, including physic-chemical properties and enzyme activities, which are important for subsequent crops (Urbatzka et al., 2009; Preissel et al., 2015). Numerous bacteria have been known to flourish in the rhizosphere, which is mostly due to the supply of nutrients and the platform supplied by plant residues (Mendes et al., 2013; Bakker et al., 2012; Sugiyama and Yazaki, 2012). In this study, the rhizosphere bacterial communities for the tobacco crop (with pre-crops of soybean, maize and tobacco) were studied using both culture-dependent physiological profiling (Biolog-Eco) and culture-independent 16S rRNA metagenomics (454 pyrosequencing). Using the Biolog-Eco plates, we found that the bacterial activity and diversity in soil from tobacco rotations (soybean and maize) were higher than in the tobacco monocropping soil (*Fig. 1* and *Table 1*). This result was consistent with the findings of Yue (2013) that continuous monocropping in tobacco cultivation led to a decrease in soil bacterial abundance. Meanwhile, some studies have found that rotation systems stimulate soil microbial biomass, enzyme activities and functional diversity and abundance in the soil community structure (Orr et al., 2012; Insam et al., 2015; Plaza et al., 2004). Thus, we speculated that pre-crops of soybean and maize affected the rhizospheric bacterial communities of subsequent tobacco. Moreover, the results of principal component analysis suggested that the soybean-tobacco and maize-tobacco rotation treatments had similar rhizospheric bacterial communities, whereas the continuous cropping of tobacco showed different bacterial communities. The preceding crop effect of grain legumes providing nitrogen has been demonstrated (Hauggaard-Nielsen et al., 2009). As a consequence, this effect varies the bacterial communities for subsequent crops in the soil. In addition to legumes, many broad-leaved crops or summer cereals produce similar rotational benefits (Preissel et al., 2015). The bacterial activity and diversity data showed no significant differences between the soybean-tobacco and maize-tobacco rotation systems, although maize and soybean leave different residues in the field after the crops have been harvested. These results suggested that the plant species planted in the current year was more important in determining the bacterial community in soils than the pre-crop species.

The 454 pyrosequencing analyses revealed 57,084 sequences obtained from the three soil libraries in this study, and *Proteobacteria*, *Acidobacteria* and *Actinobacteria* were the most abundant phyla, which generally agreed with several previous studies reporting that these phyla are capable of having various effects on plant health, including both beneficial and pathogenic interactions (Lee et al., 2008; Berendsen et al., 2012; Li et al., 2016). The rarefaction curves for these samples did not reach the asymptote. Therefore, the deeper sequencing may be required to avoid underestimation of microbial diversity in our samples. But the coverage values for all libraries were not too low (i.e. over *C*. 85%), and provide a basis for comparison with other studies. Indeed, our coverage values were largely over reported in oil-contaminated sediment (C=61%-83%). Our rarefaction curves based on 454 pyrosequencing did not saturate but we consider, on the basis of the above comparisons, that the majority of the bacterial diversity in our samples was correctly sampled.

Significant differences in bacterial community composition systems were observed between tobacco rotations and tobacco monocropping. The largest shifts in relative abundance were found for *Proteobacteria* and *Acidobacteria*. The increase in the relative abundance of *α-proteobacteria* in the soybean-tobacco rotation soil was driven by the increases in the genera *Rhodoplanes*, *Sphingobium* and *Phenylobacterium* (*Fig. 7*), suggesting that these genera are enriched by soybean as a pre-crop. The pH was the highest in the soybean-tobacco (7.02) soil compared to the tobacco monocropping (6.60)

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and maize-tobacco (6.87) soils. Previous research has demonstrated that *α-proteobacteria* and *γ-proteobacteria* showed positive relationships to soil pH (Rousk et al., 2010). The phylum *Chloroflexi* is ubiquitous in various soil samples, and its relative abundance was frequently higher than the abundance of *Bacteroidetes* (Roesch et al., 2007). This finding was further supported by the changes in our study. The relative abundance of *Acidobacteria* increased significantly in the maize-tobacco rotation soil compared to the soybean-tobacco soil. The abundance of the phylum *Acidobacteria* correlates with the soil pH (Hartman et al., 2008; Jones et al., 2009), and acidobacterial subgroups 1 and 2 decreased with increasing pH (Lauber et al., 2009), which is in consistent to our study. The biological functions of *Acidobacteria* are not well known because most microorganisms of this phylum have not been cultured (Yamada and Sekiguchi, 2009). However, as recent study found that *Acidobacteria* are capable of degrading plant litter in soils (Eichorst et al., 2011), the presence of maize residues in the maize-tobacco rotation soil may have contributed to the observed differences. Symbiotically fixed N may contribute to the nutrient availability, soil structure, and microbial activities of subsequent crops in rotation via the N mineralization of plant residues (Urbatzka et al., 2009). The relative abundance of *Actinobacteria* was strongly shaped by the pre-crop, and a significant increase was observed in the soybean-tobacco rotation soil compared to the tobacco monocropping soil. *Actinobacteria* members play an important role in the degradation of recalcitrant compounds and prevent infection with pathogenic microorganisms by secreting various antibiotics (Nour et al., 2003). In addition, previous studies have demonstrated that the *Bacillaceae_Bacillus* genus (affiliated with *Firmicutes*) was primarily involved in biological control against different soil-borne pathogens (Pal et al., 2004). Since the *Bacillaceae_Bacillus* genus showed higher relative abundances in soil from tobacco rotations with (soybean and maize) than in tobacco monocropping soil. The higher abundance of *Bacillaceae_Bacillus* in the tobacco rotation systems may contribute to disease suppression, and soybean as a pre-crop resulted in the highest bacterial community diversity and activity compared with maize and tobacco. Such results suggest that the soybean-tobacco rotation system, from a biological perspective, may represent a good strategy for enhancing microbial diversity and maintaining soil biological fertility.

Conclusion

Our research shows that the bacterial communities in the soybean-tobacco and maize-tobacco rotation soils are diverse, and various members can apparently be attributed to the pre-crops (compared to the corresponding bacterial communities in the tobacco monocropping system soil). The results obtained from the present field investigations, by using Biolog-Eco platess and next-generation sequencing from the three treatments, showed that (i) the bacterial activity and diversity in soil from tobacco rotations (soybean and maize) were higher than in tobacco monocropping soil; (ii) there were increased relative abundances of *Actinobacteria* and *Bacteroidetes*, particularly of the genera of *Rhodoplanes*, *Adhaeribacter*, and *Bacillaceae_Bacillus* in the soybean-tobacco and maize-tobacco rotation soils compared with the tobacco monocropping soil.

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Conflict of interest. The author confirms that this article content has no conflict of interest.

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USE OF BLACK SEED (*NIGELLA SATIVA* L.) OIL IN THE MANAGEMENT OF HYPERTENSIVE AND HYPERLIPIDEMIC INDIVIDUALS OF DISTRICT MUZAFFARABAD, AZAD KASHMIR, PAKISTAN

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Abstract. Hypertension and hyperlipidemia are two main causes of cardiovascular diseases with rapidly increasing pervasiveness worldwide. Complimentary alternative medicines provide an effective choice to these highly prevalent global health issues. *Nigella sativa* has diverse range of traditional and pharmacological potential with established safety profile. This study was carried out in 163 mild-moderate hypertensive and hyperlipidemic patients. Patients were selected randomly of both genders. The patient's age ranges between 20-65 years. This clinical study was conducted to evaluate the clinical effect of *Nigella sativa* virgin oil in hypertension and hyperlipidemic patients of the selected region and also assess its antioxidative potential. The results conferred that *N. sativa* has significant (< 0.05) effect on controlling hypertension and hyperlipidemia as compared to standards used. Moreover, *N. sativa* conferred excellent antioxidant potential as compared to other commercially available edible oils.
Keywords: *alternative medicines, pharmacological, antioxidants, virgin oil, edible oils*

Introduction

Hypertension is the major cause of cardiovascular diseases (CVD) which is significantly increasing day by day and according to an estimate by the end of 2025 it will go up to 1.4 billion of the world's adult population (Kearney et al., 2005). Hypertension is defined as a systolic blood pressure greater than 140 mmHg and/or diastolic 90 mmHg or greater, and any record of serum lipid abnormality is defined as hyperlipidaemia. There are number of known cardiovascular disease, renal disease, liver disease, obesity, high cholesterol and stressful conditions; an unknown reasons of high blood pressure (essential hypertension). Hypertension is a silent killer disease as it may go undiagnosed for years and when detected had developed chronic heart disease, cardiovascular disease, diabetes mellitus, renal disease, etc. However, if properly diagnose and treat would reduce the morbidity and mortality (Schuman and Emerson, 1998). Hypertension in long terms impairs the renal function and resulted to failure in

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majority of the cases (Klag et al., 1996) and these all causes cardiovascular complication (Rostand et al., 1991). Clinical studies demonstrated that a reduction in hypertension can control 42% risk of stroke and 14% coronary heart disease (Hobbs, 2004). In the struggle of modernization and exposure to globalization the population of low income countries greatly affected their lives to pursue their high living standards. Although some get well succeed status but many deprived in hands of cultural and social adjustments that may cause increase in hypertension. Hypertension has long been thought of Western world disease but now it is equally the part of poor countries. In addition to organic causes lifestyle play major role in this regard. The lifestyle of many people has become more westernized. Several studies hypothesized its contribution to hypertension in urban populations when compared to rural populations (Ma et al., 2012). Within the non-communicable diseases hypertension plays leading role posing threats of disabilities (WHO, 2013) and this is because of unawareness to these conditions by a high number of hypertensive individuals (Kayima et al., 2013). In a study of prevalence of hypertension, in low and middle income countries the blood pressure found in more than half of population higher than in US (Fuentes et al., 2000). Although hypertension is the cause of mortality and morbidity in world (Rodger et al., 2004) but stroke in urban East-African countries is five times higher than in Britain (Walker et al., 2000).

Nigella sativa is an annual herb belonging to family *Ranunculaceae* with huge medicinal potential. Its seeds and seeds extracts has been used medicinally for centuries especially in Mediterranean region, Middle-East and Southeast Asia (Rchid et al., 2004; Najmi, et al., 2008). It has tremendous traditional and pharmacological potential in curing a wide range of ailments particularly hypotension (Aqel, 1992), hypoglycaemia (Bamosa et al., 1997; Meral et al., 2001; Bamosa et al., 2010), oxidative stress (Burits and Bucar, 2000) and cardio protective (Tasawar et al., 2011). *N. sativa* plant has strong antihypertensive effect that significantly lowered down blood pressure and cholesterol (Dehkordi and Kamkhah, 2008). Reinhart et al. (2008) also reported its hypotensive, hypercholesterolemic, hypoglycaemic and antioxidative activities. *N. sativa* significantly reduces intracellular cholesterol by regulating LDL, HDL and triglycerides blood levels (El-Dakhakhany, 2000). Obesity is the main cause of metabolic syndrome (Vega, 2001). BMI is also an important indicator of metabolic syndrome (Najmi et al., 2008). *N. sativa* showed a significant reduction in the body weight when administered to a experimental model of rats (Zaoui et al., 2002). More than 100 bioactive compounds had been reported in the *N. sativa* seeds (Ramadan, 2007). The therapeutic potential of medicinal plants is mainly due to the antioxidative properties of some active components (El-Saleh et al., 2004). The seeds of *N. sativa* contains two active components in its oils i.e. Thymoquinone and dihydrothymoquinone, revealed enormous potential of free radical scavenging capabilities (Khalife and Lupidi, 2007). These biological active compounds considered largely as chemo protective (Badary et al., 1999 and Badary et al., 2007), gastro protective (El-Abhar et al., 2003; Kanter et al., 2005) and immuno protective (Gilani et al., 2004). The foods rich in fats after absorption through intestine intensify the hepatic detoxification, enhance lipid peroxidation and resulted debris cause cellular modifications. Cholesterol in the blood vessels also constructs fibrosis plaque along the walls. These plaques are atherosclerotic proliferation of the extra cellular matrix formed due to extensive biochemical and molecular changes within the vessels (Glass and Witztum, 2001; Tiwari et al., 2008). Oxidation by free radicals is an imperative incident cause aging and human diseases.

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The antioxidant screening of plants and their phytochemicals through comparing commercial antioxidants could help to find new source of expected innate antioxidants. *N. sativa* and its active components have tremendous potential of nutraceutical and pharmaceutical applications. This needs to be explored through more clinical studies in metabolic syndrome that is the challenge of future to medical professionals. The present study was undertaken to explore probable antioxidant potential of *N. sativa* found in its different parts which is responsible for lowering high blood pressure, serum cholesterol and plasma sugar. The data on the evaluation of *N. sativa* clinically and as antioxidant are scanty. This was the first ever study for validating the therapeutic potential of *N. sativa* in the state of Azad Kashmir, Pakistan.

Methodology and Materials

Patient Selecting Criterion

Patients with mild-moderate hypertension were selected from outpatient departments (OPD) of Abbass Institute of Medical Sciences (AIMS) Muzaffarabad and from medical camps organized through AIMS cardiology department. Before starting the study an approval from hospital ethical committee was taken. After explaining all the outcomes of the treatment and taken written consent from all participants (*Supplementary Figure 3*), pathological history was recorded through questionnaire (*Supplementary Figure 1*). During selection every aspect of patient interest focussed so that satisfies his convenience in perfection of better compliance. A total of 180 patients were registered for this clinical study which was divided in to two groups, each comprising 90 individuals. All patients were selected from the same geographical area randomly with a male and female ratio of 43:57. Selection of Patients were based on category, Mild-Moderate Hypertension, Systolic Blood Pressure 130-159 mmHg; Diastolic Blood Pressure 80-99 mmHg, Abdominal obesity (Waist circumference): >102 cm in males and >88 cm in females, Total Cholesterol: ≥ 200 mg/dL, Serum Triglycerides: > 150 mg/dL, LDL: > 120 mg/dL, HDL: < 40 mg/dl (male) or HDL < 50 mg/dL (female). Serum Glucose: ≥ 110 mg/ dL.

Patient's weight and height were measured for Body Mass Index calculation BMI (Kg/m²). Patient's systolic and diastolic blood pressure was recorded by using mercury Sphygmomanometer (Tycos Japan). Fasting blood samples (Venus) were taken in the hospital pathological laboratory and analysed for all biochemical tests using principal biochemistry analyzer and enzyme assays. Adverse events report proformas (*Supplementary Figure 2*) to report any adverse event and laboratory proformas (*Supplementary Figure 4*) to file all base line tests reports were prepared. A copy of each was given to patient for record. Patients were divided randomly including male and female's age ranged from 20-65 years into *N. sativa* and standard statin groups. One group was started with *N. sativa* oil treatment while second on statin standard treatment (atorvastatin 10 mg tablet once a day) and metformin 500 mg tablet one twice a day added to diabetics after taking a baseline data of all biochemical parameters. All the patients were strictly advised to follow the guide lines regarding physical activities, diet and maintained it regularly in routine life style. *N. sativa* seeds virgin oil (NsVO) administered orally twice a day in a dose of 0.5 ml before breakfast and going to bed for sleeping at night. During the study period patients were closely monitored through telephone calls, personal visits and every fortnight calls on hospital visits for blood

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pressure examination. After 45 days patient's blood samples taken in the hospital pathological laboratory and data were analyzed statistically by using paired t-test.

The Antioxidant Assay

Antioxidant potential of *N. sativa* and edible oils was evaluated by using free radical scavenging DPPH assay (2,2-diphenyl-1-picrylhydrazyl) as described by Amarowicz et al. (2004). The absorbance was recorded at 517 nm through ultrospec-4000 (Pharmacia-LKB) UV-visible spectrophotometer. The radical scavenging activity was expressed as $IC_{50.}$ Ascorbic acid was used as standard. The percentage scavenging activity was calculated by using the following formula:

$$\text{Percentage Inhibition} = \frac{(\text{A control} - \text{A extract})}{(\text{A control})} \times 100$$

where A control is the absorbance of the control reaction, A extract is the absorbance of the test sample.

Results and Discussion

The study was undertaken with the aim to compare the antihypertensive and antihyperlipidemic effect of *N. sativa* virgin oil and standard statin treatments with their respective baseline data's in selected area. For this purpose 180 patients was screened out. Out of 180 patients 163 comply while 17 did not due to personal reasons. Statistical analysis showed that *N. sativa* has significant (P-value = < 0.05) effect on controlling hypertension (Systolic Bp P=3.079e-07; Diastolic Bp P=2.136e-06) and hyperlipidemia (Total cholesterol P = 2.2e-16, LDLc P = 2.2e-16, HDLc P = 4.739e-12 and TGs P = 0.05706) as compared to standard group (Systolic Bp P=5.505e-07; Diastolic Bp P = 1.005e-07; Total cholesterol P = 4.805e-13, LDLc P = 4.803e-10, HDLc P = 4.049e-06 and TGs P = 1.441e-07) through its antioxidative activities (Table 4, 5). The patients receiving *N. sativa* treatment after 45 days the mean systolic (154.35 mmHg) and diastolic (95.20 mmHg) blood pressure at baselines was reduced to 142.80 mmHg and 88.15 mmHg respectively (*Table 1; Fig. 1, 3*). Among the 83 patients 59 (71%) showed a significant decreased in blood pressure, 13 (16%) increased and 11 (13%) showed no effect of the treatment (*Fig. 2*). In comparison to the 2nd group of standard treatment after 45 days the mean systolic (153.60 mmHg) and diastolic (95.18 mmHg) blood pressure at baselines was also reduced to 146.60 mmHg and 90.30 mmHg respectively (*Table 1, Fig. 4*) but it was less than *N. sativa* group. Among the 80 patients receiving standard treatment 46 (57%) showed a decreased in blood pressure, 16 (20%) increased and 18 (23%) showed no effect of the treatment (*Fig. 3*). The percent effect of *N. sativa* (71%) at individual levels was also better compared to standard group (57%). Various traditional and animal studies report the promising hypotensive action of the seeds and extracts of *N. sativa*. Rahman et al. (1990) described the antihypertensive effect of the methanolic seeds extracts of *N. sativa* in the normal and adrenaline induced hypertension. Dehkordi and Kamkhah (2008) suggested that 200 mg *N. sativa* seeds extract twice daily for 8 weeks significantly lowered down the systolic and diastolic blood pressure.

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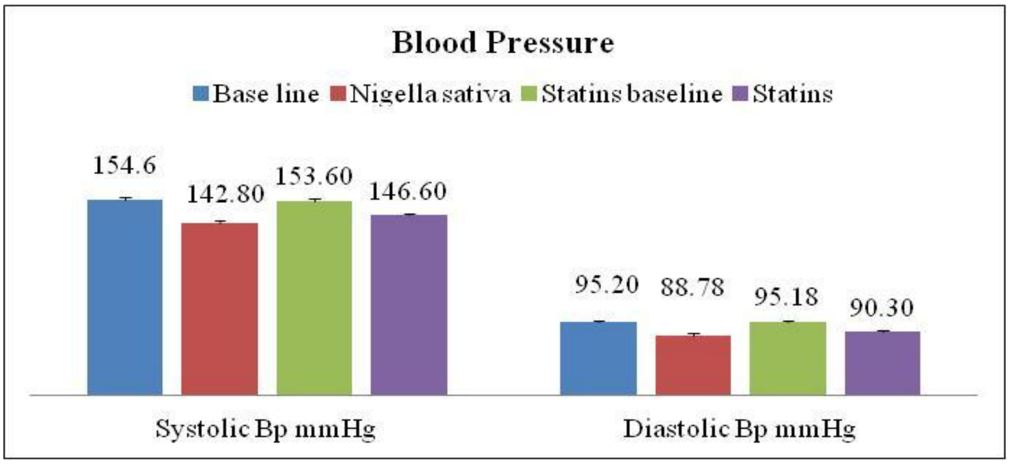

Figure 1. *Mean comparison of N. sativa patients vs. standard statin at baseline after 45 days treatment*

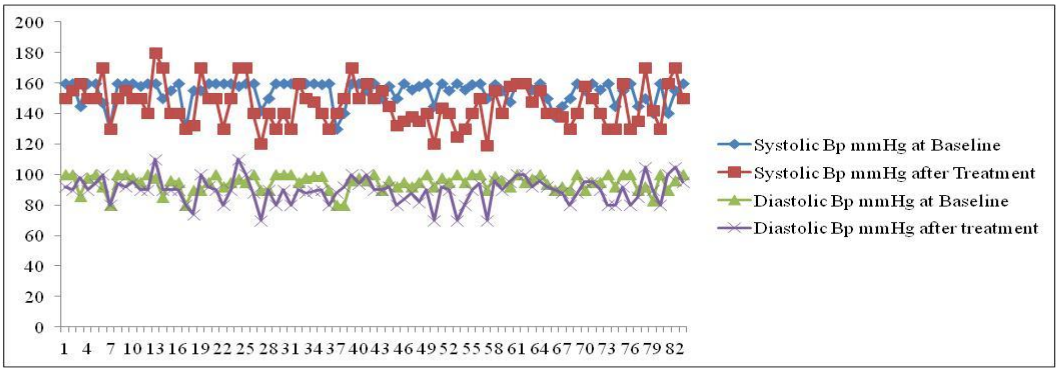

Figure 2. *Pattern of systolic and diastolic blood pressure of the individuals after N. sativa treatment at baseline*

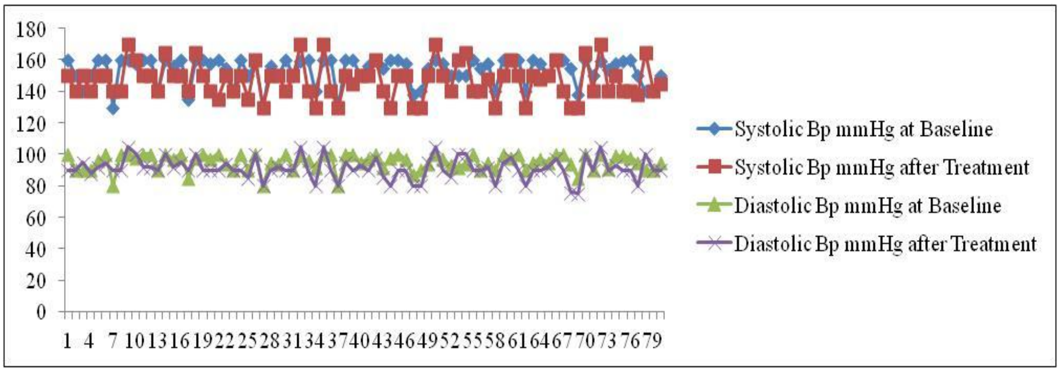

Figure 3. *Pattern of systolic and diastolic blood pressure of the individuals after standard treatment at baseline*

The *N. sativa* treatment showed a considerable reduction in the total cholesterol (~218-205 mg/dl), LDLc (~135-122 mg/dl) and triglycerides (~204-194 mg/dl) levels at their baselines as compared to the standard treatment which were ~222-215 mg/dl, ~136-130 mg/dl and ~190-180 mg/dl respectively. There was found a significant

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reduction in the mean total cholesterol (P = 2.2e-16) and LDLc (P = 2.2e-16) in *N. sativa* group as compared to standard group (P = 4.805e-13 and P = 4.803e-10). The effect of seeds extract in animal model (for 12 weeks) has been evaluated by Zaoui et al. (2002) which showed a decrease in the levels of cholesterol, triglycerides and blood glucose. Dekhordi and Kamkhah (2008) depicted that 100 or 200 mg twice daily doses of *N. sativa* significantly reduced the total and LDL cholesterol in a dose dependent manner. Pourghassem-Gargari et al. (2009) and Nader et al. (2010) reported that if black seeds used in diet supplements can effectively decrease the total cholesterol (43.7 %), LDLc (42.8 %) and TGs (34.9 %) after one month of treatment as compared to control. The improvement in HDLc is although low (37.7 3-39.50 mg/dl), however better than in case of standard therapy (39.18-39.5 mg/dl). The results are in agreement reported by Najmi et al. (2008) and Le et al. (2004).

Reduction in BMI has positive effect on obesity. The treatment with *N. sativa* showed the reduction of BMI (26.22-25.52 kg/m²) as compared to standard group (27.90-27.38 kg/m²) however waist in both cases (~92-91 and ~98-97 cm) were comparable (*Table 1*). The reduction in triglyceride levels were also found same in both groups (204-194 mg/dl and 190-180 mg/dl). Similar results were reported by Najmi, et al. (2008) while working with *N. Sativa.* Datau et al. (2010) found a reduction in the body mass index when used *N. sativa* seeds in a dose of 1.5 g/day for three months in obese individuals. Although their study conferred that the reduction in BMI and waist was not much significant but it had good impact on improving cholesterol. Moreover, In the *N. sativa* group the HDLc was raised 37.73-39.50 mg/dl while in standard group the value was 39.18-39.50 mg/dl which showed a significant (P = 4.739e-12) augment due to the *N. sativa* treatment. It has also been observed the high impact of the *N. sativa* treatment on the reduction of fasting blood glucose levels (~150-128 mg/dl) in comparison to the standard treatment (~147-144 mg/dl) at baseline (*Table 1; Fig. 4, 5*). The studies of Bamosa et al. (2002) and Najmi, et al. (2008) also supported the findings of the present study on total cholesterol, LDL, HDL, Triglyceride and fasting blood sugar.

Table 1. *Mean comparison of N. sativa vs. standard treatment at their respective baselines*

Biochemical Parameters	**Control vs. NsVO**		**Control vs. Statin**	
	Before Treatment Means Value	After Treatment Means Value	Before Treatment Means Value	After Treatment Means Value
Systolic Bp (mmHg)	154.35	142.80	153.60	146.60
Diastolic Bp (mmHg)	95.20	88.78	95.18	90.30
BMI (Kg/m²)	26.22	25.52	27.90	27.38
Waist (cm)	92.00	91.33	98.00	97.13
T. Cholesterol (mg/dl)	218.25	204.80	221.65	215.13
LDL (mg/dl)	134.63	122.10	135.75	130.10
HDL (mg/dl)	37.73	39.50	39.18	39.50
Triglycerides (mg/dl)	203.70	193.73	189.80	180.23
BSF (mg/dl)	149.73	128.54	146.73	143.92

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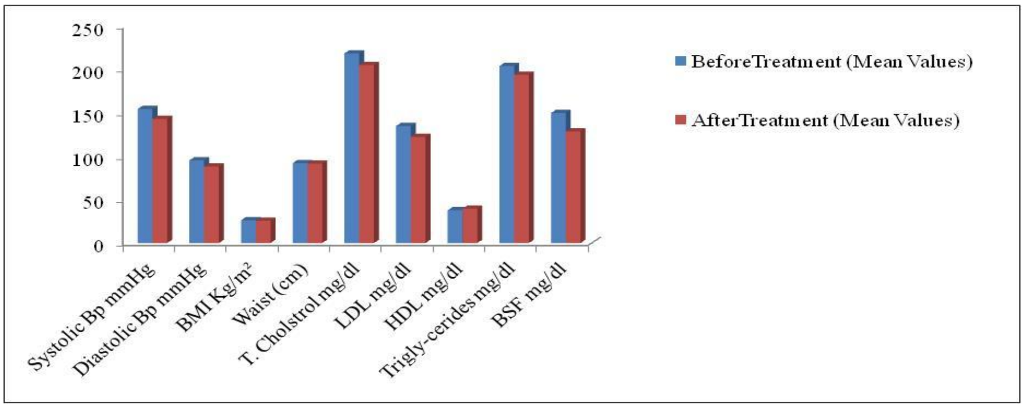

Figure 4. *Mean comparison of N. sativa patients vs. base line after 45 days treatment*

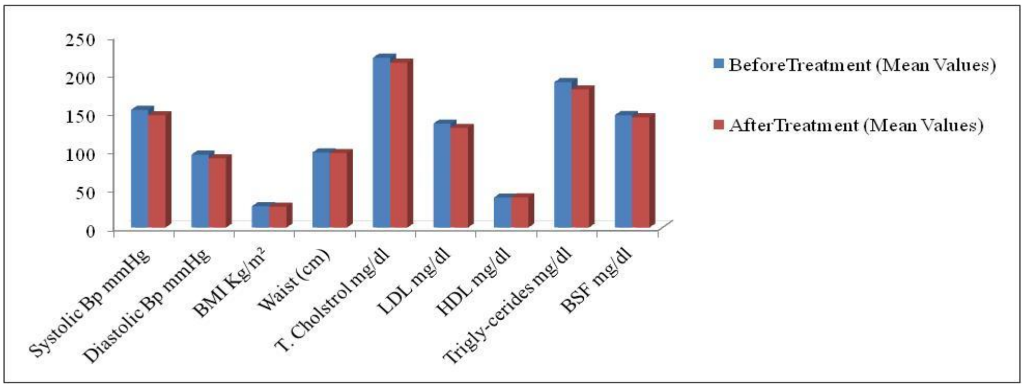

Figure 5. *Mean comparison of standard vs. baseline after 45 days treatment*

Furthermore, when cholesterol and sugar levels were undertaken resulted lowered down along with blood pressure which also controlled due the fact that an increase in blood pressure found to be linked with increased cholesterol and sugar levels (Siok-Koon et al., 2009). The systolic and diastolic blood pressure significantly lowered down with the increased in duration of the *N. sativa* treatment. The systolic blood pressure was decreased to ~134 mmHg after 90 days and ~127 mmHg after 180 days of *N. sativa* treatment at baseline of ~155 mmHg followed by decrease in diastolic blood pressure as ~83 mmHg and ~76 mmHg after 90 and 180 days at baseline of ~94 mmHg respectively (*Table 2; Fig. 6*). In case of standard treatment the systolic (~153 mmHg) blood pressure was decreased to ~145 and 138 mmHg after 90 and 180 days while the diastolic (~94 mmHg) was decreased to ~90 and ~84 mmHg after 90 and 180 days respectively (*Table 3; Fig. 7*). Similarly patients receiving *N. sativa* showed a remarkable reduction in the mean total cholesterol, LDLc and triglycerides with the increase in HDLc after six month of treatment. The *N. sativa* group significantly reduced the cholesterol (199 and 186 mg/dl), LDLc (113 and 99 mg/dl), triglyceride (168 and 154 mg/dl) and fasting blood sugar (112 and 94 mg/dl) after 90 and 180 days of treatment at baselines of 220 mg/dl, 136 mg/dl, 191 mg/dl and 150 mg/dl respectively. The HDLc was increased to 40.80 mg/dl and 42.13 mg/dl at the baseline of 39.05 mg/dl after 90 and 180 days of *N. sativa* treatment respectively (*Table 2; Fig. 6*).

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Table 2. *Mean comparison of N. sativa group after 45, 90 and 180 days treatment at baseline*

Biochemical Parameters	Control	After 45 Days	After 90 Days	After 180 Days
Systolic Bp (mmHg)	155.07	142.80	134.18	127.28
Diastolic Bp (mmHg)	94.3	88.78	83.23	76.35
BMI (kg/m²)	26.65	25.52	25.93	25.55
Waist (cm)	91.75	91.33	90.15	89.13
T. Cholesterol (mg/dl)	219.7	204.80	198.53	186.03
LDL (mg/dl)	135.9	122.10	112.70	99.20
HDL (mg/dl)	39.05	39.50	40.80	42.13
Triglycerides (mg/dl)	190.8	193.73	168.00	154.25
BSF (mg/dl)	149.73	128.54	111.73	93.73

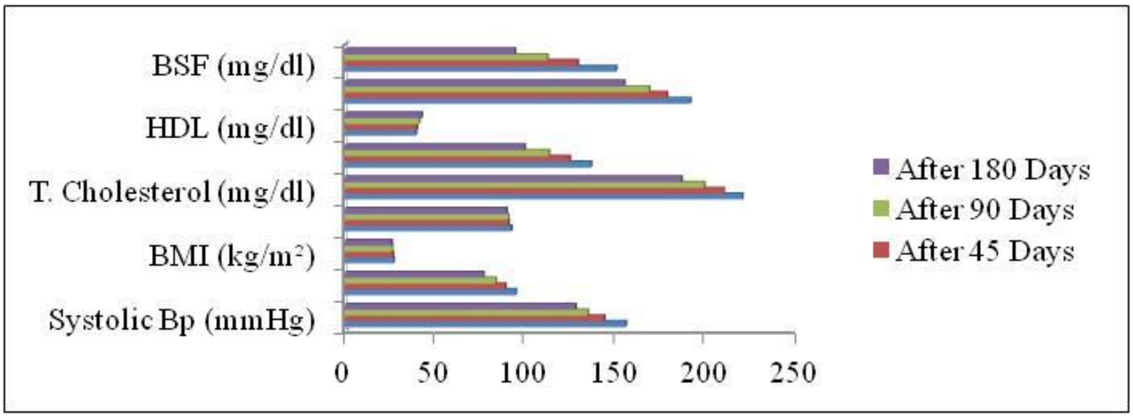

Figure 6. *Mean comparison of N. sativa group after 45, 90 and 180 days treatment at baseline*

Table 3. *Mean comparisons of standard group after 45, 90 and 180 days treatment at baseline*

Biochemical Parameters	Control	After 45 Days	After 90 Days	After 180 Days
Systolic Bp (mmHg)	153.15	146.60	145.0	137.8
Diastolic Bp (mmHg)	95.15	90.30	90.15	84.25
BMI (kg/m²)	27.48	27.38	26.89	26.53
Waist (cm)	96.65	97.13	95.8	95.1
T. Cholesterol (mg/dl)	226.95	215.13	218.4	210.07
LDL (mg/dl)	137.27	130.10	126.8	121.15
HDL (mg/dl)	38.75	39.50	39.82	40.67
Triglycerides (mg/dl)	195.07	180.23	178.22	159.57
BSF (mg/dl)	146.73	143.92	127.08	129.27

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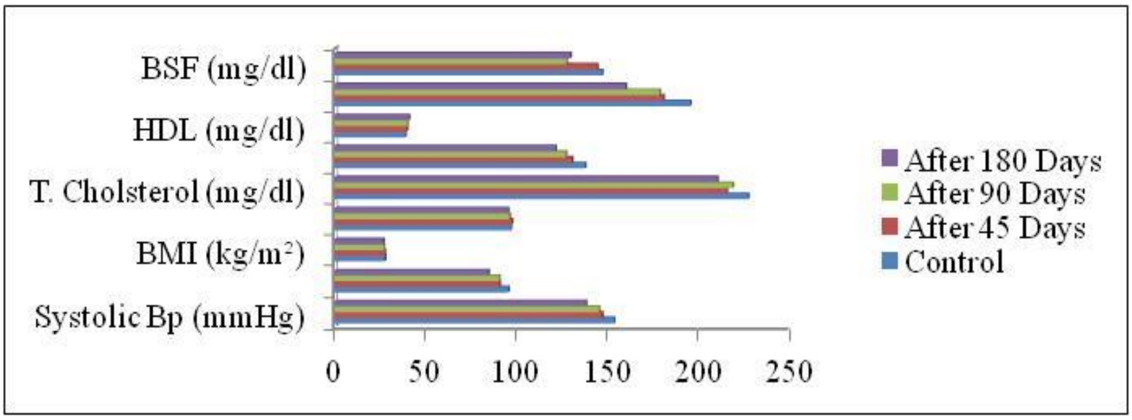

Figure 7. *Mean comparisons of standard group after 45, 90 and 180 days treatment at baseline*

Table 4. *Comparison of variables before and after N. sativa treatment using paired t-test*

Variables of *N. sativa* Group	T-values	DF	P-values	95% Confidence Interval	Remarks
Systolic Bp (mmHg)	5.57	82	3.079 e-07	5.656962 11.933400	Significant
Diastolic Bp (mmHg)	5.10	82	2.136 e-06	3.123754	Significant
BMI (Kg/m²)	9.57	82	5.284 e-15	0.4335806 0.6611182	Significant
Waist (cm)	5.56	82	3.215 e-07	0.6967115 1.4719632	Significant
T.Cholesterol (mg/dl)	11.12	82	2.200 e-16	9.130463 13.110501	Significant
LDL (mg/dl)	11.015	82	2.200 e-16	9.072539 13.072039	Significant
HDL (mg/dl)	8.085	82	4.739 e-12	0.8538746 1.4111856	Significant
TG (mg/dl)	1.93	82	0.05706	-0.1847185 12.2088148	Insignificant

Table 5. *Comparison of variables after and before standard treatment using paired t-test*

Variables of Standard Group	T-values	DF	P-values	95% Confidence Interval	Remarks
Systolic Bp (mmHg)	5.4524	79	5.505 e-07	3.97632 8.54868	Significant
Diastolic Bp (mmHg)	5.8629	79	1.005 e-07	2.617235 5.307765	Significant
BMI (Kg/m²)	0.9611	79	0.3394	-0.2577192 0.7389692	Insignificant
Waist (cm)	5.0999	79	2.279 e-06	0.4725213 1.0774787	Significant
T. Cholesterol (mg/dl)	8.6449	79	4.805 e-13	4.464578 7.135422	Significant
LDL (mg/dl)	7.1002	79	4.803 e-10	4.039113 7.185887	Significant
HDL (mg/dl)	4.9542	79	4.049 e-06	0.2392906 0.5607094	Significant
TG (mg/dl)	5.7769	79	1.441 e-07	5.866225 12.033775	Significant

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The systolic and diastolic Bp significantly (P-value = < 0.05) decreased when treatment of *N. sativa* was continued for another 45 days. The systolic and diastolic blood pressure was raised from ~143-148 mmHg and ~88-94 mmHg respectively when that group of patients was treated with placebo however, the total cholesterol was increased from ~200-209 mg/dl and LDLc ~102-125 mg/dl while no significant change was recorded in the levels of HDLc and triglycerides. Moreover a little change conferred in BMI (25.52-25.33 kg/m²) and waist circumference (91.32-90.12 cm) while blood sugar was raised from 128 – 146 mg/dl (*Table 6*).

Table 6. *Comparison of variables before and after placebo treatment using paired t-test*

Variables of Placebo Group	T-values	Degree of freedom	P-values	95% Confidence Interval	Remarks
Systolic Bp (mmHg)	2.6223	39	0.01239	1.126098 8.723902	Significant
Diastolic Bp (mmHg)	4.5873	39	4.562e-05	3.032939 7.817061	Significant
BMI (kg/m²)	3.2084	39	0.002669	0.04517891 0.19932109	Significant
Waist (cm)	-1	39	0.3235	-0.15113455 0.05113455	Insignificant
T.Cholesterol (mg/dl)	5.8299	39	8.924×10^-07	4.163177 8.586823	Significant
LDL (mg/dl)	5.4011	39	3.509e-06	8.897783 19.552217	Significant
HDL (mg/dl)	2.6234	39	0.01236	-0.26565178 -0.03434822	Significant
TG (mg/dl)	1.1905	39	0.241	-1.799843 6.949843	Insignificant
BSF (mg/dl)	6.9145	25	3.011e-07	12.42255 22.96206	significant

The antioxidant activity of the extracts of all lines of *N. sativa* was assessed by using DPPH assay and IC_{50} value recorded was tabulated in Table 7. Among seven lines tested, the N1 sample was found to be potent (*Table 7; Fig. 8*). When compared different solvents extracts, the ethyl acetate was found to be a potent solvent for the extraction of potential compound with IC_{50} values for N1 (0.0022), N2 (0.0033), N3 (0.0030) and N7 (0.4476) while methanol, chloroform and acetone extracts showed least activity (*Table 7; Fig. 8*). Due to the factual antioxidant potential of seeds of *N. sativa*, a detail screening of all parts was carried out. Here also the N7 line was found to be potent. The IC_{50} value recorded for ripen seeds was 0.000415 followed by unripe (green seeds) i.e. 3.510731 as compared to the standard used (*Table 8; Fig. 9a* and *3c*). Other parts were found to be rubbished (*Table 8; Fig. 9b*). Vinha et al. (2005) reported that variation in antioxidant potential among all parts of same genotype was due to variation in secondary metabolites in different parts or may be due to extraction procedures, which is adopted for the extraction of active constituents. Commercially available edible oils were also screened with the purpose to compare their potential with selected genotype. It was found that *N. sativa* had great antioxidant potential as compared to the edible oils tested (*Table 9; Fig. 9d*). According to Skerget et al. (2005) sometime sampling of the same plant from the same ecological zone may deviate to the potential behavior because of mishandling of sampling procedures that could immediately oxidized the active metabolites and the antioxidant ability of the plants.

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Yoruk et al. (2010) also reported significant antioxidant potential of *N. sativa* preventing oxidative stress by scavenging reactive oxygen species. The current results were in harmony with the previous studies.

In the present findings of clinical studies the use of *N. sativa* virgin oil (N7) revealed a significant reduction in the blood pressure and other biochemical parameters. The evidence from literature also supports the findings that the antioxidants protect the body against the development of atherosclerosis and provide putative hypotensive effects (Rohdewald, 2002; Paulis and Simko, 2007). Antioxidants contained melatonin (Flavonoids) can effectively be used in reducing blood pressure, lipid profile, body weight and plasma glucose (Hussein et al., 2007). Antioxidants have strong impact of reducing the blood pressure which is also supported by a recent study (Rezzani et al., 2010) that described the protective role of antioxidants against the initiation of atherosclerosis in hypertensive animal model. Houghton et al. (1995) reported that the biologically active constituents of *N. sativa* had potent quenching capabilities for free radicals. On the basis of present findings it is conferred that the poor antioxidant effects in treating various pathological conditions are more or less due to improper dosage, methods of extraction, selection of the seeds cultivar and the selection of the seeds lots. So it is suggested that only an intellectual approach could succeed in achieving the desired results while implementing any treatment of plant material consisting these oxidants. Moreover the oxidative stress promptly impregnate its harmful effects on the arterial vasculature that require long time to correct in a smoothly manner. Allopathic drugs although provide to some instant relieve to the inflammation of the endothelial linings of the vasculature but could not be the sole solution to the whole imploratory process of atherosclerosis.

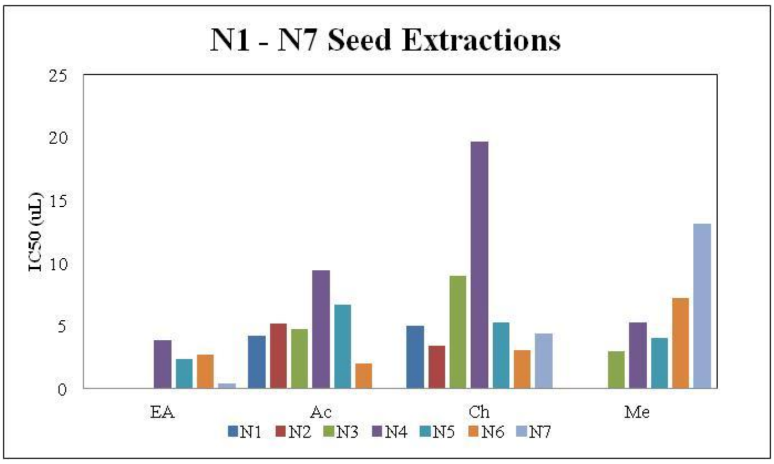

Figure 8. *Comparison of antioxidant activities of N. sativa seeds in four solvent extracts of seven lines in different concentrations and IC50 values*

Conclusion

On the basis of the current investigations it is concluded that *N. sativa* seeds and oil should be considered routinely in the management of diabetic and hypercholesterolemic disorders as remedy was found to be potent hypotensive, antihyperlipidemic and hypoglycaemic with strong antioxidative activities without any adverse effect. *Nigella sativa* proved to be the best alternatives remedial source to cure these fatal ailments with

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remarkable benefits of many other concomitant cures. However more investigations are needed to isolate bioactive compounds that are responsible of managing high blood pressure and other biochemical disorders.

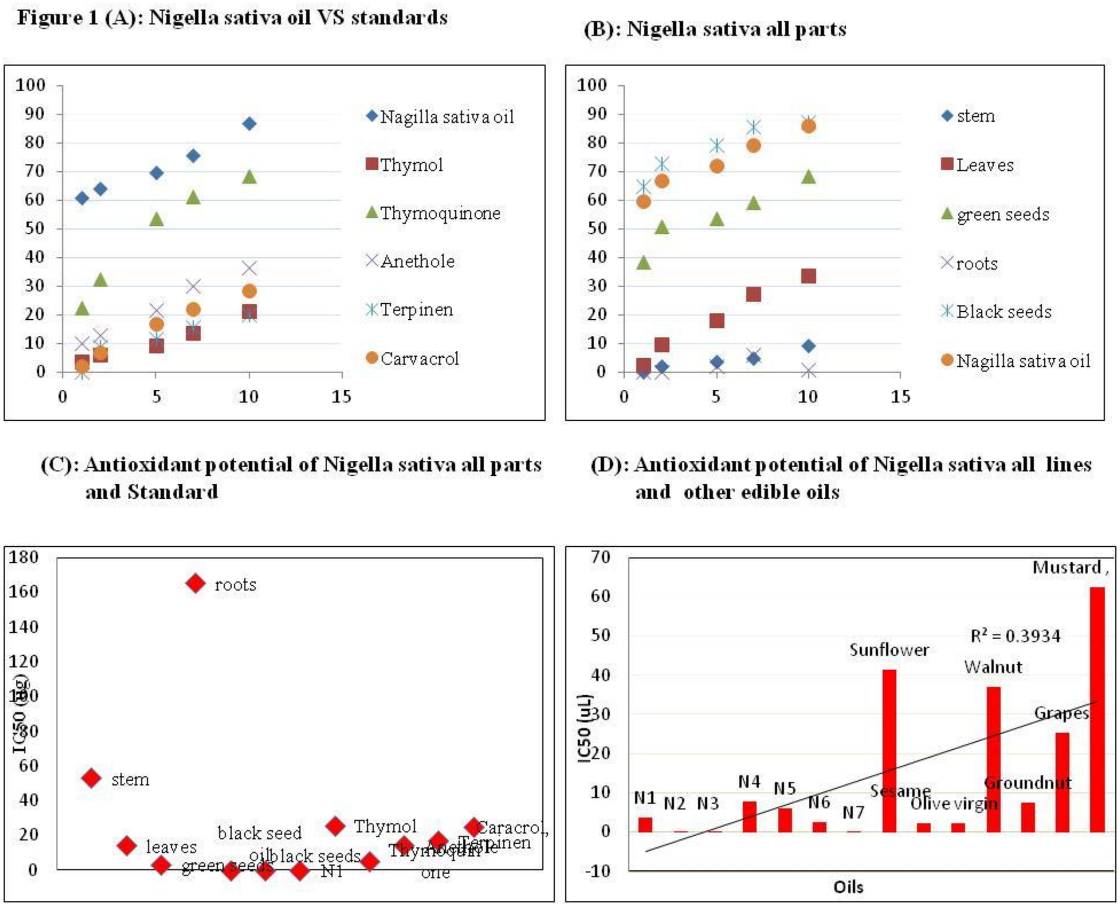

Figure 9. *Antioxidant activities of N. sativa seeds oil VS standards (A), all parts (B), all parts VS standards (C) and all seven lines VS other edible oils*

Table 7. *Comparison of antioxidant activities of N. sativa seeds in four solvent extracts of seven lines in different concentrations and IC_{50} values*

Samples	Solvent	1 µl%	IC_{50}
N1	Ethyl acetate	51.12	0.002266
N1	Acetone	25.89	4.199698
N1	Chloroform	27.45	5.0361
N1	Methanol	45.48	0.00217
N2	Ethyl acetate	68.99	0.00335
N2	Acetone	16.22	5.204309
N2	Chloroform	36.28	3.474909
N2	Methanol	59.79	0.00255
N3	Ethyl acetate	49.89	0.002035
N3	Acetone	29.94	4.721764
N3	Chloroform	30.69	8.978789
N3	Methanol	28.87	2.963774
N4	Ethyl acetate	51.04	3.859536
N4	Acetone	09.33	9.405586
N4	Chloroform	16.32	19.64961

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N4	Methanol	16.00	5.326029
N5	Ethyl acetate	36.17	2.412749
N5	Acetone	21.43	6.666936
N5	Chloroform	25.99	5.312122
N5	Methanol	38.92	4.090206
N6	Ethyl acetate	53.18	2.721577
N6	Acetone	37.11	2.038345
N6	Chloroform	29.98	3.098866
N6	Methanol	47.00	7.267184
N7	Ethyl acetate	55.89	0.447679
N7	Acetone	28.65	0.003108
N7	Chloroform	21.08	4.366061
N7	Methanol	34.00	13.11741

Table 8. *Antioxidant potential of all parts of N. sativa versus its five standards used*

S. No.	Plant parts and Standards used	1 µl	IC_{50}
1	Stem	00.08	53.24247
2	Leaves	02.55	14.32170
3	Green Seeds	38.56	3.510731
4	Roots	00.01	165.3653
5	Ripened Seeds	65.08	0.000415
6	Milled Oil	59.89	0.000359
7	Thymol	03.66	25.75068
8	Thymoquinone	22.56	5.423880
9	t-Anethole	10.29	14.15704
10	Terpinen	00.03	16.88444
11	Carvacrol	02.11	24.97511

Table 9. *Antioxidant activities of other edible oils available in the market and IC50 values*

Samples	Solvent	$1\ \mu l_{\%}$	IC_{50}
Oil	Sunflower	00.08	41.3329
Oil	Sesame	46.99	2.273876
Oil	Olive virgin	48.33	2.160575
Oil	Walnut	04.44	36.98957
Oil	Groundnut	00.01	7.588462
Oil	Grapes	00.91	25.44962
Oil	Mustard	23.76	62.50398

The beneficial effects of *N. sativa* in diabetic's subjects with fasting sugar, LDL, triglycerides, total cholesterol, BMI and high blood pressure were investigated and found highly significant (P = < 0.05) although the *N. sativa* was comparable to standards in short term but in longer therapy *N. sativa* revealed excellent role in controlling biochemical disorders. Maintaining serum glucose, serum cholesterol and high blood pressure at optimum reduces atherosclerosis and subsequently the risk of coronary heart diseases, a major threat to diabetic and hyperlipidemic patients. The potent antioxidative activities of *N. sativa* played a central role in functional optimization of biochemical parameters.

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APPENDIX

Name of Participant				
Address				
Contacts				
Gender	☐ Male	☐ Female	☐ Age	
Age Group	☐ 20-35yrs	☐ 36-50yrs	☐ 51-65yrs	
Marital Status	☐ Single	☐ Married	☐ Divorced/Widow	
Living Place	☐ Urban	☐ Rural	☐ Remote	
Education	☐ Primary	☐ Secondary	☐ College	
	☐ University			
Department	☐ Govt.	☐ Semi-Govt.	☐ Private	
Occupation	Job Nature:____________		Position:____________	
No. of Dependants	☐ 1-3 Persons	☐ 4-6 Persons	☐ 7-9 Persons	
Exercise/activities	☐ 0 activity	☐ 1-1	☐ 2-2	☐ 3-3
	☐ 4-4	☐ 5-5		
Diet Habit	☐ Normal	☐ Mix	☐ Fatty/Spicy	
Living Environment	☐ Pleasant	☐ Tough	☐ Depressive	
Job Environment	☐ Easy	☐ Compatible	☐ Stressed	
Transportation	☐ Walking	☐ Public Transport	☐ Official Conveyance	
Sleeping Habits	☐ Comfortable	☐ Delayed	☐ Difficult	
Family Life	☐	☐	☐	

Supplementary Figure 1. *Questionnaire used for the subjects in clinical studies*

Registration for the Clinical Studies

I am explained all the benefits and outcomes of the treatment that will have to be given me during this clinical studies. I agreed voluntarily basis and promise to be loyal with all its treatment protocol.

Name: ______________________________ ID: __________________________

Male or Female: _________________________ Date of Birth: ________________________

Signature: ____________________________ Contact #: __________________________

Witness: ____

Diagnosed Indications

____

Responsible Clinician

Name: ____________________________ Position: ____________Tel#:______________

Date:________Time:_______

Supplementary Figure 2. *Proforma used for subjects consent participating in clinical study*

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Serious Adverse Event Report

If there is suspected any Serious Adverse Event (SAE) related to the treatment, register all the details in the Performa below and please call immediately 03455894220.

Responsible Clinician

Name:________________________ Position: _________________Tel#: _____________

Patient Details. Date:________Time:________

Pt. Name: ________________________________ Patient ID: _________________________

Male or Female: ____________________________ Date of Birth: ____________________

Treatment#:________________________________ Dosage: _______________________

Allocated Date: _____________________________ Duration used: __________________

Adverse Event Details

Event: ____

Time & Date of onset: [| |] End time and date: [| |]

Mild to Moderate	Severe	Life Threatening	Hospitalization	Persistent or Recovered

Other; ___________ (Not covered by categories but, in the investigator opinion, should be considered serious).

How you suspect the adverse event to be related to this treatment?________

How you rate this event treatment related from 0-100% [ ]

Patient Name/Signature ________________ Date:_______________

Supplementary Figure 3. *Proforma used for subjects reporting serious adverse events*

	Clinical Investigations			
				Date: …………..
1	**Blood Pressure:**	Sistolic ………..	Diastolic: …………………………..	
		(References)		
2	**Lipid Profile:**	Lower Limit	Upper Limit	
	Cholesterol: ……………………………..	3.6 mmol/l	6.5mmol/l	
	Triglyceride: …………………………..	≤1.7mmol/l	≤1.7mmol/l	
	LDL: ……………………………….	2mmol/l	3.4mmol/l	
	HDL: …………………………………..	0.9mmol/l	2.2mmol/l	
3	**Serum Glucose:**			
	Random: ………………………………	6.6mmol/l	10mmol/l	
	Fasting:……………………………… ………	3.8mmol/l	6.1mmol/l	
	Creatnine Clearance:…………………………………………………..………………………………… ….			
	BMI:………………………………… …….	Weight:……… ……	Waist:……… …..	Height:……… ….

Patient consent
Do you agree for clinical trial?

Name & Sig

Supplementary Figure 4. *Proforma used for subjects clinical investigations*

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COMPARISION OF HPLC AND BIOASSAY METHODS FOR ANALYSIS OF SULFOSULFORUN RESIDUES IN WHEAT FIELD SOIL

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Abstract. Sulfosulfuron, a new sulfonylurea herbicide, is used to control certain broadleaf and grass weed species in wheat fields. Herbicides, when applied to the field may leave unwanted residues in the soil. Bioassay and chemical assay techniques are main methods for monitoring herbicides levels in agricultural soils. Thus, a set of field and greenhouse experiments was established to evaluate the reliability and accuracy of HPLC and bioassay techniques to detect sulfosulfuron residues in the soil of the wheat field. The experiment was conducted in a randomized complete block design with three treatments in three replications for each treatment in 2014-2015 and 2015-2016 growing seasons at Marvdasht location. Herbicide treatments included: sulfosulfuron (apirus) at 26.6 and 53.2 g a.i. ha^{-1} rates and no-herbicide control. Herbicide was sprayed at the end of tillering stage of wheat in a field experiment. Samples were collected randomly with an auger from 0-10 cm depth at different times i.e. 0, 3, 10, 20, 30, 60, 90 and 125 days after herbicide spray. The bioassay experiment showed that root parameter was more sensitive to sulfosulfuron as compared with the shoot. According to the results, among the studied plants, garden cress was recognized as the most sensitive plant to different rates of sulfosulfuron herbicide. At recommended (26.6 g a.i. ha−1) and double (53.2 g a.i. ha^{-1}) rates of application sulfosulfuron residues were detected up to 60 and 90 days after treatment by HPLC and bioassay techniques in both years, respectively. According to the bioassay and HPLC techniques at two rates of application, residues decreased with time in 2014 and 2015.
Keywords: *herbicide, bioassay, high-performance liquid chromatography, half-life, root parameter*

Introduction

Most farming systems extensively use herbicides. When applied to the field, herbicides control targeted weeds and leave unwanted residues in the soil that are harmful ecologically (Haney et al., 2000; Derksen et al., 2002). Despite the great efficacy of herbicide for controlling weeds, its residual effect should be taken into account for the safety of the environment. Ideal herbicides should have good efficacy and minimum adverse effects on crops, ecology and the environment (Faheed and Abd-Elfattah, 2007).

The most important crop in Iran is wheat which can be considered as the main food for the entire population (Vahedi-Sheikhhasan et al., 2012; Rahman et al., 2008). The second crops such as cotton, corn, soybean, canola and sunflower are sown after wheat is harvested as rotation crops. Good weed control is a necessary component of wheat yield (Hasanuzzaman et al., 2009). To do this, in wheat fields, the application of herbicides especially sulfonylurea is common. In the 1970s, sulfonylurea herbicides were introduced, which are a class of herbicides that were used as control chemicals for the majority of broad-leaved weeds and common grasses in agriculture (Coly and

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Aaron, 1999; Guibiao et al., 2006). These herbicides reside in the acetolactate synthase enzyme which is the main enzyme in the biosynthesis of branched-chain amino acids and is characterized by a high herbicidal activity that leads to application at low rates (Nystrom and Blanck, 1998; Brown, 1990; Ye et al., 2003). Sulfonylurea herbicides apply post-emergence in cereal specially wheat therefore there was short period between herbicide application and next planting which caused adverse effects on crops in rotation with wheat (Menne and Berger, 2001). Of sulfonylurea herbicides, sulfosulfuron (1- (4,6-Dimethoxypyrimidin-2-yl) -3- (2-ethylsulfonylimidazol [1,2-a] pyridin-3-ylsulfonyl) urea), has been suggested for weed control in wheat fields (Nurse et al., 2007). Sulfosulfuron which is a new sulfonylurea herbicide, is used to control weeds in different agricultural crops and wheat (Parrish et al., 1995). These new herbicides have an extensive range of soil residual characteristics that must help with meeting specific agricultural requirements (Maheshwari and Ramesh, 2007). Sulfonylurea herbicides consist of 3 distinct parts: a sulfonylurea bridge, an aryl group and an S-triazine group. Of the reasons they were quickly accepted include a very low animal toxicity, low application rate and a broad-spectrum weed control. The removal of sulfonylurea herbicides can be done in three ways in the environment and these methods are: chemical hydrolysis, microbial degradation and photodegradation. Potential transformation in sulfonylurease is the cleavage of the sulfonylurea bridge yielding sulfonamide, S-triazine and Striazinurea bridge contraction and ring opening which forms triurets (Sarmah et al., 1998).

Soil pH, organic matter, moisture and temperature are the major factors that affect sulfonylurea chemical hydrolysis and microbial degradation. In soil, sulfonylurea hydrolysis is primarily pH dependent and its rate goes up with decreasing soil pH (Beyer et al., 1988). Recent studies have proven a similar pH dependence of sulfosulfuron hydrolysis in soil incubation research (Saha and Kulshrestha, 2002). The study of Pusino et al. (2003) which was done on the desorption and the adsorption of a sulfonylurea herbicide triasulfuron in three different soils, revealed that, pH was the main cause which affected the absorption level and also showed that, adsorption in soils, was negatively correlated with pH. The highest amount of adsorption was measured in soils which had low pH and high organic carbon content. In specific conditions like highly alkaline soils with low temperatures and organic matter contents in dry regions, sulfonylurea herbicides might persist at phytotoxic rates in soils to have negative impacts on sensitive crops in the subsequent season (Stork, 1995).

The main methods for monitoring herbicide levels in agricultural soils are Bioassay and Chemical assay techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC) (Tchan et al., 1975; Johnson et al., 2005; Watson and Checkel, 2005). Because the application of chemical techniques was limited due to higher costs, the bioassay technique is a useful tool to detect residues herbicides. Moreover, chemical assay techniques are able to determine herbicide residues rates but cannot determine that these rates are really toxic to plants (Szmigielski et al., 2008). Alonso-Prados et al. (2002) showed that the European Commission Guidance Document on Residue Analytical Methods has accepted and suggested bioassays as suitable screening tests that can be good for excluding the occurrence of low levels of residues of phytotoxic compounds in soils. Many researchers used bioassay methods to discover herbicide residues in soil (Alonso-Prados et al., 2002; Blacklow and Pheloung, 1991; Gunther et al., 1993; Hernandez-Sevillano, 2001; Stork and Hannah, 1996).

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The chemical extraction method cannot distinguish between available and unavailable herbicide. Also, a chemical extraction will not show an indication of potential plant response. As a replacement to chemical extraction methods, bioassay is the most accurate method available for the detection and quantification of sulfonylurea residues in soil (Beyer et al., 1988). Based on the study of Ferris and Haigh (1992), bioassay is the level of a plant's response to the total herbicide residue in the soil at a site-specific location.

As far as we know, little or no research has been done to detect sulfosulfuron residues in the soil. The objectives of this research were to: 1) determine half-life of sulfosulfuron in soil, 2) determine the most sensitive plant species to sulfosulfuron, 3) and to compare analytical and bioassay techniques for monitoring sulfosulfuron herbicide level in soil.

Material and Methods

Field Experiment

The study were conducted in two consecutive years 2014-2015 and 2015-2016 at Marvdasht location (Fars province, Iran) that situated in 29°52'43.46" N (Latitude DMS) and 52°49'13.99" E (Longitude DMS) (*Fig. 1*). Before sowing, soil samples were taken from 0 to 30 cm depth and physicochemical properties of the experimental site were determined (*Table 1*). The field at the test site had lain fallow the year before the start of our study. To prepare the seedbed, deep plowing (20-25 cm) with a moldboard plough were carried each year in autumn, followed by disking in the spring. The soil fertility was increased by using diammonium phosphate (18-46-0 N-P-K) and urea at the rate of 250 and 150 kg a.i. ha^{-1}, respectively, each year in spring before planting. Moreover, 200 kg a.i. ha^{-1} N (as urea) was added at the 6-8 leaf growing stage of wheat along with irrigation. The wheat hybrid "Singles Cross 704" was sown at desired density (7 plants a.i. m^{-2}), seeds spaced 18.5 cm apart in rows spaced 20 cm apart on 15 May 2014 and 22 May 2015. The experimental design at two years was a randomized complete block with three replications. Sulfosulfuron was applied as POST (Post-emergence application) at 26.6 g a.i. ha^{-1} (recommended rate) and 53.2 g a.i. ha^{-1} (double rate) with a knapsack sprayer using flat fan nozzle at four-leaf stage in each year. Three plots were sprayed with water without any herbicide and considered as the control plots. The experimental fields were 9 plots 10 m×3 m size with a buffer of 0.5 m between adjacent plots to avoid spray overlap.

Table 1. *Physicochemical traits of soil in the location of experiment*

Soil texture	Sand (%)	Silt (%)	Clay (%)	N (%)	P (ppm)	K (ppm)	$CaCO_3$ (%)	OM (%)	pH	EC (dS m^{-1})
Clayloam	34.6	35.0	30.4	0.015	3.4	240.0	12.0	0.36	7.5	0.45

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Figure 1. *Location of Marvdasht in Fars province*

Sampling and Storage

Soil samples were collected randomly throughout each plot from 0–10 cm depth using a tube auger 0 (2 h), 3, 10, 20, 30, 60, 90 and 125 days after treatment. Samples were air dried and ground to pass through a 2-mm sieve, mixed thoroughly and subsamples were taken from each plot for the bioassay and HPLC studies. Samples were kept in a deep freezer at -20 °C until they were subjected to the bioassay and HPLC analysis.

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Greenhouse Experiment

Bioassay Technique

Determination of test plant for bioassay study: Greenhouse experiments were conducted to select the most sensitive plant species to evaluate sulfosulfuron residues in soil. Eight different plant species including lentil (*Lens culinaris* Medik.), garden cress (*Lepidium sativum* L.), mung bean (*Vigna radiata* L.), cucumber (*Cucumis sativus* L.), corn (*Zea mays* L.), sugar beet (*Beta vulgaris* L.), canola (*Brassica rapa* L.) and chick pea (*Cicer arietinum* L.) were screened by measuring their shoot and root responses to soil-incorporated. The pots (10 cmID ×10 cm length) were filled with 500 g of soil and treated with sulfosulfuron at 5, 10, 20, 50, 75 and 100% of recommended rate (26.6 g a.i. ha^{-1}), five pre-germinated seeds of each test-plant were planted at 2–3 mm depth in each pot. The pots were arranged on greenhouse in a completely randomized design with four replicates along with untreated control pots. The pots were sub-irrigated as needed during the experiment. The plants were uprooted to measure the sulfosulfuron effects on plants root and shoot length at 15 days after planting (Paul et al., 2009). During the experiment, temperature and relative humidity were fluctuated between 18–25 °C and 58–85%, respectively.

Preparation of standard curve by bioassay and analysis of field samples: To establish a standard curve, the samples soils (500g) fortified with sulfosulfuron concentration (5, 10, 20, 50, 75 and 100% of recommended rate (26.6 g a.i. ha^{-1}) were filled in the plastic pots with four replicates along with untreated controls. Five pre-germinated garden cress seeds (*Lepidium sativum* L.) were sown as the indicator species in each pot. The experiment was conducted under greenhouse conditions as completely randomized design. The pots were irrigated as needed to retain the soil moisture. 15 days after sowing, all plants were uprooted and washed carefully with water to remove the soil. Then, root length for garden cress plants were measured as sensitive parameter and the percentage reduction in root length relative to the control plants was calculated for each concentration. A standard curve was drawn by plotting the percentage root inhibition on a vertical linear (y) axis versus the corresponding sulfosulfuron concentration on horizontal logarithmic (x) axis. The soil samples which were taken at different intervals from wheat field were placed in pots, and five pregerminated garden cress seeds were planted into these pots as described above. After 15 days, all plants were uprooted and percentage root inhibition was calculated for each interval. Finally, the herbicide residues were determined under field conditions by fitting the data correspond to percentage root inhibition into the regression equation (Paul et al., 2009).

HPLC Technique

Chemicals

Analytical-grade sulfosulfuron (97% purity) with chromatographically (TLC and HPLC) pure was supplied by Sigma–Aldrich (Steinheim, Germany). The chemical structure of sulfosulfuron herbicide is given in *Figure 2*. All solvents, such as acetonitril and dichloromethane were HPLC grade and purchased from Merck (Darmstadt, Germany).

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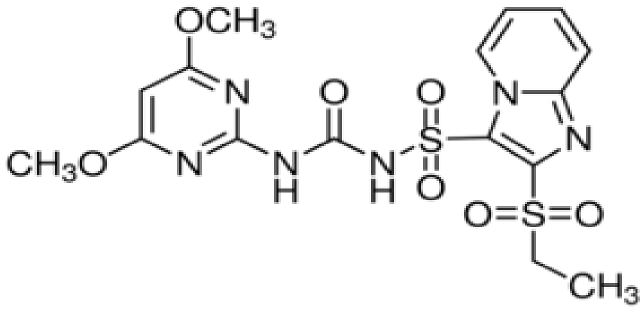

Figure 2. *The structure of sulfosulfuron herbicide (1-(4,6-Dimethoxypyrimidin-2-yl)-3-(2-ethylsulfonylimidazol[1,2-a]pyridin-3-ylsulfonyl)urea)*

Soil Extraction and Clean-up

Fifty g of soil were put in a conical flask and 50 ml of acetonitrile and ammonium carbonate (9:1 v/v) was added to it. The filled flask was shaken for 30 min and its contents were partitioned. We separated and filtered the upper organic layer. The soil was shaken with 50 ml of acetonitrile: ammonium-carbonate (9:1 v/v) once again. next, the upper organic phase were separated and mixed with the first fraction. The mixed extract was gathered and dried at 40°C on a rotary evaporator and then decreased to 20 ml. Next, it was dissolved in 50 ml of saline water (1 M NaCl). The solution was put in the separating funnel and partitioned twice with 50 ml of dichloromethane. The mixed dichloromethane extract was then collected and passed through anhydrous sodium sulfate which was packed in a 30 cm column to remove all traces of moisture. Then filtrate was collected, pooled and almost completely dried at 40 °C on a flash evaporator. At the end, the residue was dissolved in 2 ml of HPLC grade acetonitrile and filtered through 0.45 μm Millipore system before being injected into the HPLC system (Saha et al, 2003).

Preparation of Standard Curve by HPLC

A stock solution (1000 μg a.i. mL^{-1}) of sulfosulfuron was prepared in acetonitrile and different concentrations including 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μg a.i. mL^{-1}) of sulfosulfuron were made by diluting the stock solution. A volume of 15 μL of each standard solution was injected into the HPLC and the peak area measured. Each run was repeated twice and the calibration curve was created by drawing the known concentrations of sulfosulfuron on the x-axis and the average peak area corresponding to each concentration on the y-axis.

Apparatus Conditions

Sulfosulfuron was detected with a HPLC equipped with a photodiode array detector, a C18 column (250 mm × 4 mm ID), a mobile phase of acidic water + acetonitrile + o-phosphoric acid, 20 + 80 + 0.1 (v/v), a flow rate of 1 mL a.i. min^{-1}, a UV – detector set at a wavelength of 225 nm. A volume of injection was 15 μL for each standard solution and the retention time was 2.9 min. Samples were filtered before injection by a 0.45 μm membrane using a millipore filtration syringe.

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Data Analysis

Data analysis was performed by using the Statistical Analysis System (SAS). Means were compared by the least significant difference (LSD) test at $\alpha = 0.05$. The three-parameter sigmoidal model was used to determine dissipation time (DT50) of sulfosulfuron herbicide in Marvdasht location: $f = a/(1 + \exp(-(x - x_0) / b))$, where the parameter a is maximum dissipation of herbicide, b is the slope of the curve around the X_0 and X_0 donates time required for 50% dissipation. Figures were drawn by using the microsoft excel software.

Results

Determination of Test Plant for Bioassay Study

The bioassay experiment showed that root parameter was more sensitive to sulfosulfuron as compared with shoot. As shown in *Figures 3* and *4*, the percentage root length reduction was within the approximate range of 40 to 71% for the bioassay plants, while the shoot length inhibition for bioassay crops was lower (25 to 60%). Since the roots are directly exposed to the herbicide residues, it seems that the influence of the herbicide residues on roots is higher than shoots. These results revealed the differential sensitivities of plant species to sulfosulfuron. Among the eight crops tested, garden cress had the maximum percentage decrease in root length with root length inhibition of approximately 71% at recommended rate which was followed by lentil, canola, sugar beet, cucumber, mung bean, chick pea and corn (*Fig. 3*). Thus, garden cress was considered as the test species for planting in the field soil samples in bioassay experiments and the percentage root length inhibition was found as the standard parameter. This finding is consistent with findings of Paul et al. (2009). Szmigielski et al. (2009) developed the sugar beet bioassay to study the soil-incorporated sulfentrazone.

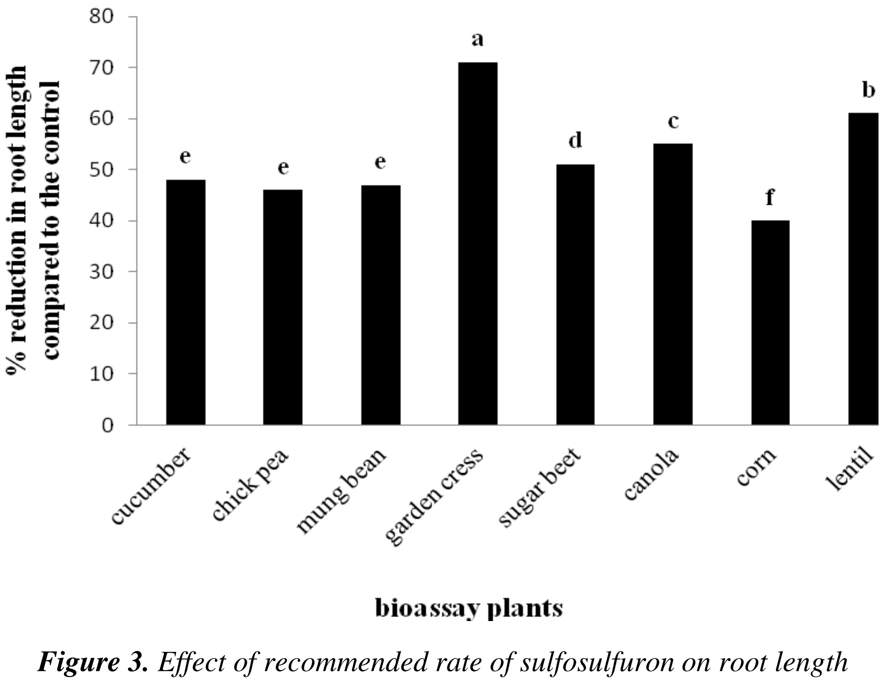

Figure 3. *Effect of recommended rate of sulfosulfuron on root length in different bioassay plants*

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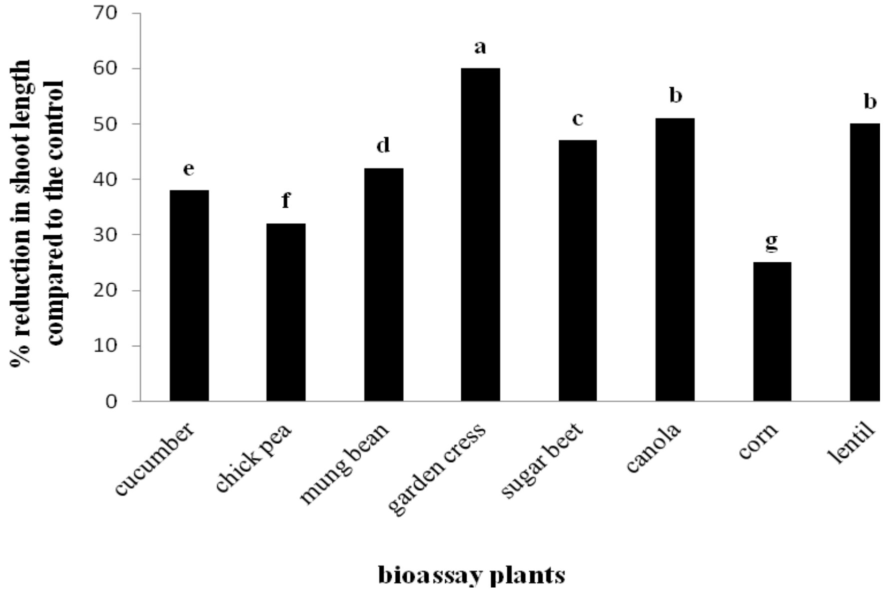

Figure 4. *Effect of recommended rate of sulfosulfuron on shoot length in different bioassay plants*

Calibration Curve

To establish a standard curve, soil samples treated with six different levels of sulfosulfuron (5, 10, 20, 50, 75 and %100 of recommended rate) were filled in pots with four replicates for each concentration and five pregerminated garden cress seeds (in each pot) were planted as the indicator species. After 15 days of planting, the percentage reduction in root length of garden cress plants versus different concentrations of sulfosulfuron was measured and the respective calibration curve was drawn. At the recommended rate of application (26.6 g a.i. ha^{-1}), the best regression equation fitted was linear as Y = 7.3781X + 4.0174 with R^2 = 0.97 (*Fig. 5*). At the double rate of application (53.2 g a.i. ha^{-1}), the best regression equation fitted was linear as Y = 4.4272X+13.494 with R^2 = 0.98 (*Fig. 6*). 15 days after planting, the percentage inhibition of garden cress root length of the field samples, were entered into the standard equation to determine the herbicide concentration that was bioavailable.

For HPLC, a series of standard solutions containing different concentrations of sulfosulfuron (0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µg a.i. mL^{-1}) were prepared in acetonitrile by diluting the stock solution (1000 µg a.i. mL^{-1}). 15 microliters of each concentration was injected and the calibration curve was established based on the sulfosulfuron concentration versus corresponding peak. The best fitted regression equation was Y = 177947X + 40771 with R^2 = 0.99.

Recovery and Limit of Quantification (LOQ)

The accuracy and precision of the method was performed by recovery study. Recovery study was conducted for soil by extraction and analysis of five replicates at three different levels (0.01, 0.05 and 0.1 mg a.i. kg^{-1}). The average recoveries of sulfosulfuron in soil were varied from 78 to 85%. The limit of quantification of sulfosulfuron was detected to be 1 µg a.i. kg^{-1}.

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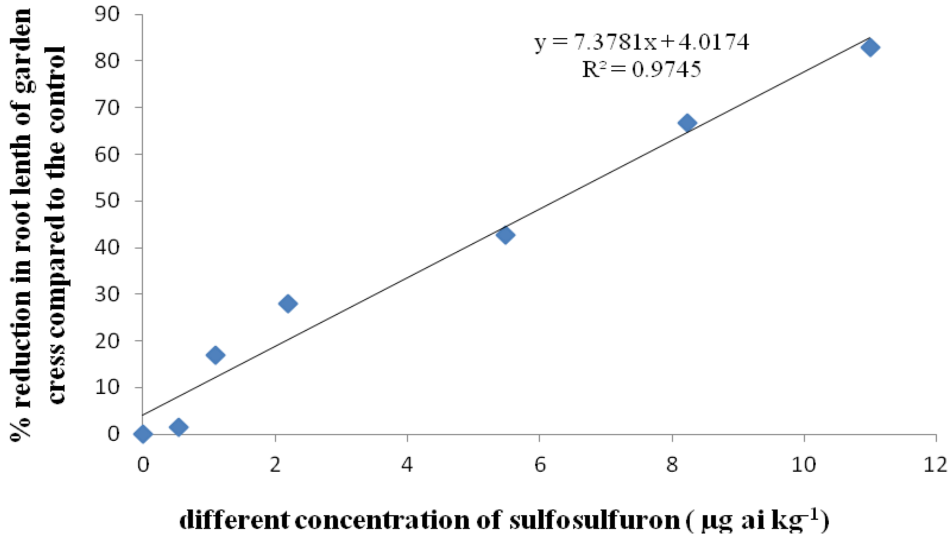

Figure 5. *The percentage reduction in root length of garden cress plants versus different concentrations of sulfosulfuron (26.6 g a.i. ha^{-1})*

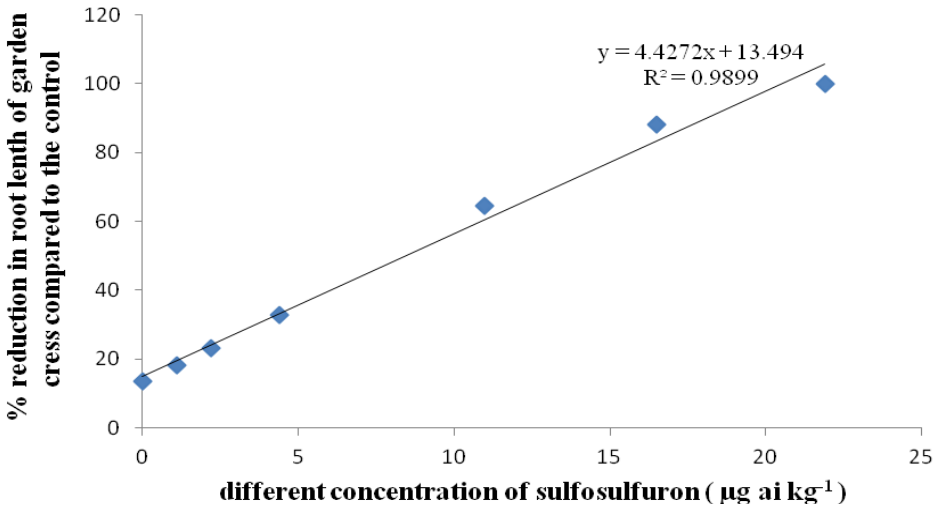

Figure 6. *The percentage reduction in root length of garden cress plants versus different concentrations of sulfosulfuron (53.2 g a.i. ha^{-1})*

Persistence of Sulfosulfuron in Wheat Field Soil

HPLC Method

The results of extracted herbicide concentration in the soil for different rates are shown in *Table 2*. Results of the sulfosulfuron dissipation in the wheat field soil displayed that initial concentration of sulfosulfuron residues in the surface soil (0-10 cm) were 9.8 and 10.1 µg a.i. kg^{-1} for recommended rate (26.6 g a.i. ha^{-1}) and 19 and 18.5 µg a.i. kg^{-1} for double rate (53.2 g a.i. ha^{-1}) application in 2014 and 2015, respectively. 3 days after application (DAA), dissipation of sulfosulfuron was 17.19 and 18.1 % for 26.6 g a.i. ha^{-1} and 15.37 and 17.19 % for 53.2 g a.i. ha^{-1} application in 2014 and 2015, respectively. 20 days after application, the residues decreased to 5.2 and 6.8

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µg a.i. kg^{-1} for recommended rate and 10.6 and 12.4 µg a.i. kg^{-1} for double rate in 2014 and 2015, respectively. After 60 days of application, the residues decreased to 1.5 and 1.2 µg a.i. kg^{-1} with 86.35 and 89.1 % dissipation for recommended rate and 7.2 and 4.0 µg a.i. kg^{-1} with 67.24 and 81.8 % dissipation for double rate in 2014 and 2015, respectively. 90 days after application, the sulfosulfuron residues were found to be below detectable level at recommended rate. However, 1.6 and 2.1 µg a.i. kg^{-1} residues were detected at double rate in 2014 and 2015, respectively (*Table 2*). At recommended rate of application, the half-life of sulfosulfuron by the chemical extraction method were 17.28 and 19.42 days in 2014 and 2015, respectively.

Table 2. *Persistence of sulfosulfuron residues in wheat field soil at the recommended (26.6 g a.i. ha^{-1}) and double rates of application (53.2 g a.i. ha^{-1}) by HPLC*

Time (days)	**year**	**Herbicide residue remaining (± SD) (µg a.i. kg^{-1})[a]**	
		26.6 g a.i. ha^{-1}	**53.2 g a.i. ha^{-1}**
0	2014	9.8 (±0.035)[10.82]	19.0 (±0.065) [13.55]
	2015	10.1 (±0.030)[8.10]	18.5 (±0.058) [15.83]
3	2014	9.1 (±0.028)[17.19]	18.6 (±0.074) [15.37]
	2015	9.0 (±0.024)[18.10]	18.2 (±0.070)[17.19]
10	2014	7.1 (±0.037)[35.39]	NA
	2015	7.5 (±0.041)[31.75]	15.8 (±0.069)[28.11]
20	2014	5.2 (±0.032)[52.68]	10.6 (±0.054)[51.77]
	2015	6.8 (±0.029)[38.12]	12.4 (±0.060)[43.58]
30	2014	3.0 (±0.025)[72.70]	8.7 (±0.061)[59.20]
	2015	2.0 (±0.033)[81.80]	9.2 (±0.055)[63.40]
60	2014	1.5 (±0.045)[86.35]	7.2 (±0.078)[67.24]
	2015	1.2 (±0.040)[89.10]	4.0 (±0.063)[81.80]
90	2014	BDL	1.6 (±0.050)[92.72]
	2015	BDL	2.1 (±0.052)[90.44]
125	2014	BDL	BDL
	2015	BDL	BDL

[a]Average of three replicates; numbers in square brackets indicate % dissipation; NA=sample not analyzed; SD =standard deviation; BDL=below detectable level (1 µg a.i. kg^{-1}).

Bioassay Method

Data obtained in the study of sulfosulfuron persistence by the garden cress seed bioassay technique, calculated using the regression equation $Y = 7.3781X + 4.0174$, indicated that the residues of sulfosulfuron were detected up to 90 DAA in 2014 and 2015 at the recommended rate of application (*Table 3*). At day 0, the decreases in garden cress root length were 78.3 and 80.4% for recommended rate and 82.17 and 79.2% for double rate in 2014 and 2015, respectively (*Tables 3* and *4*). At the recommended rate of application, the initial deposit of 10.06 and 10.35 µg a.i. kg^{-1} in 2014 and 2015 decreased to 8.41 and 9.05 µg a.i. kg^{-1} at 10 DAA in 2014 and 2015, respectively (*Table 3*). At the double rate of application, the initial deposit of 15.51 and 14.84 µg a.i. kg^{-1} in 2014 and 2015 decreased to 12.44 and 12.76 µg a.i. kg^{-1} at 10 DAA in 2014 and 2015, respectively (*Table 4*).

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Table 3. *Persistence of sulfosulfuron residues at the recommended rate of application (26.6 g a.i. ha^{-1}) by bioassay method*

Days[a]	Year	Root length (± SD) (cm)[b]		Root inhibition (%)	Average residue (µg a.i. kg^{-1})	Dissipation (%)
		Untreated	Treated			
0	2014	13.8±1.3	3.0±0.34	78.3	10.06	8.46
	2015	11.2±0.75	2.2±1.0	80.4	10.35	5.82
3	2014	12.3±0.9	2.9±0.8	76.4	9.81	10.73
	2015	12.3±1.1	2.8±0.5	77.3	9.93	9.64
10	2014	11.5±0.7	3.9±0.6	66.1	8.41	23.47
	2015	12.0±0.4	3.5±1.0	70.8	9.05	17.65
20	2014	10.0±0.65	4.8±0.75	52.0	6.50	40.85
	2015	11.1±0.9	5.0±1.1	54.9	6.89	37.30
30	2014	10.8±0.9	7.8±0.4	27.8	3.22	70.70
	2015	13.0±0.6	8.8±1.0	32.3	3.83	65.15
60	2014	12.2±0.5	9.6±0.8	21.3	2.34	78.70
	2015	10.8±0.8	8.3±1.2	23.4	2.62	76.16
90	2014	11.0±1.3	9.5±1.1	13.6	1.29	88.26
	2015	12.4±1.0	10.1±0.7	18.5	1.96	82.16
125	2014	12.6±1.2	12.9±0.25	0	BDL	100
	2015	12.0±0.5	12.2±0.5	0	BDL	100

[a] Day after herbicide application.
[b] Average of four replicates root length for 20 plants.
SD = standard deviation.
BDL = below detectable level (1 µg a.i. kg^{-1}).

Table 4. *Persistence of sulfosulfuron residues at the double rate of application (53.2 g a.i. ha^{-1}) by bioassay method*

Days[a]	Year	Root length (± SD) (cm)[b]		Root inhibition (%)	Average residue (µg a.i. kg^{-1})	Dissipation (%)
		Untreated	Treated			
0	2014	12.9±0.67	2.3±0.34	82.17	15.51	29.43
	2015	12.5±1.3	2.6±0.6	79.2	14.84	32.48
3	2014	12.3±0.48	2.7±0.58	78.04	14.57	33.71
	2015	11.8±0.75	2.8±0.3	76.27	14.17	35.53
10	2014	10.5±1.2	3.3±0.54	68.58	12.44	43.40
	2015	11.0±1.0	3.3±0.4	70.0	12.76	41.94
20	2014	11.0±0.25	5.0±0.75	54.5	9.26	57.87
	2015	11.2±1.1	4.9±0.64	56.25	9.65	56.09
30	2014	10.8±0.9	6.2±0.4	42.59	6.57	70.10
	2015	9.5±1.2	5.1±0.54	46.3	7.41	66.28
60	2014	11.2±0.45	8.0±0.45	28.57	3.40	84.53
	2015	10.4±0.5	7.1±0.3	31.73	4.11	81.30
90	2014	11.2±1.1	8.8±0.65	21.4	1.78	91.90
	2015	11.0±1.0	8.4±1.1	23.63	2.28	89.60
125	2014	11.0±0.7	11.2±0.3	0	BDL	100
	2015	12.2±0.8	12.4±0.4	0	BDL	100

[a] Day after herbicide application.
[b] Average of four replicates root length for 20 plants.
SD = standard deviation.
BDL = below detectable level (1 µg a.i. kg^{-1}).

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In 2015, the residues of sulfosulfuron dissipated with a slow rate so that the residues were detected up to 90 DAA at the recommended rate of application whereas the residues were below detectable level at 125 DAA (*Table 3*). Moreover, the residues were detected up to 90 DAA at the double rate in both years (*Table 4*). At the recommended rate of application, Fitting data of the percentage dissipation of herbicide at different intervals to a three -parameter sigmoidal model indicated that X_0 value, which is the half-life of herbicide and estimated based on the percentage of sulfosulfuron dissipation versus different day intervals, were found to be 20.31 days in 2014 and 21.78 days in 2015 by the bioassay method (*Table 5*).

Table 5. *The three-parameter sigmoidal model f= (a/ (1+exp (-(x-x₀)/b)) to determine dissipation time (DT 50) of sulfosulfuron herbicide at the recommended rate of application (26.6 g a.i. ha⁻¹) by bioassay and HPLC techniques*

Model parameters	Technique			
	Bioassay		**HPLC**	
	2014	**2015**	**2014**	**2015**
A	89.87 (0.801)	86.85(0.920)	94.00 (0.99)	96.55 (0.94)
B	8.97 (0.290)	8.62 (0.321)	10.20 (0.29)	9.44 (0.23)
X_0	20.31(0.396)	21.78 (0.450)	17.28(01.02)	19.42(01.21)
R^2	0.97	0.96	0.98	0.96
RMSE	9.21	7.71	5.30	8.95
P-value	<0.0001	<0.0001	<0.0001	<0.0001

Values in parentheses indicate ±SE (standard error).
A = maximum dissipation of herbicide.
B = the slope of the curve around the X_0.
X_0 = is time required for 50% dissipation.

Discussion

Bioassay Analysis of Soil-Bound Herbicides

According to the bioassay experiment, root parameter was more sensitive to sulfosulfuron as compared with shoot. A root length inhibition bioassay is an effective tool to detect small amounts of phytotoxic compounds in the soil, however it may not necessarily reflect yields observed in field. Several bioassays have been developed for the detection of soil residual herbicides. A bioassay involves assessing some component of plant growth such as root length, shoot length, or yield as a function of herbicide concentrations in soil. A bioassay can be used as a quantitative procedure to determine the total amount of a certain herbicide residue present in a soil sample or to assess phytotoxicity (Sunderland et al., 1991). The application of bioassays to measure ALS inhibiting herbicides in the soil is an effective method as these compounds are potent inhibitors of root and shoot growth of susceptible plants (Brown, 1990). This method has proven useful and valid for the detection of several different herbicide residues (Szmigielska et al., 1998; Sunderland et al., 1991).

Comparison of Sulfosulfuron Persistence in Field Soil By HPLC and Bioassay Methods

At the recommended rate of application (26.6 g a.i. ha^{-1}), the initial deposit observed on day 0 was 10.06 and 10.35 μg a.i. kg^{-1} by the bioassay in 2014 and 2015 versus 9.8 and 10.1 μg a.i. kg^{-1} by HPLC in 2014 and 2015, respectively. The differences between

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residues detected by the two techniques were more obvious with time, so that on 3 DAA for recommended rate the residues detected by HPLC was 9.1 and 9.0 μg a.i. kg^{-1} in 2014 and 2015 versus 9.81 and 9.93 μg a.i. kg^{-1} detected by bioassay in 2014 and 2015, respectively. The dissipation rate of residues estimated by HPLC was more than bioassay. On 20 DAA for recommended rate, dissipation of herbicide was 40.85 and 37.30% by bioassay technique, in 2014 and 2015 versus 52.68 and 38.12% by HPLC technique in 2014 and 2015, respectively. At the recommended rate of application, sulfosulfuron residues were detected up to 60 days after treatment by HPLC method in both years, whereas residues were detected up to 90 days after treatment by bioassay method in 2014 and 2015. It has demonstrated that the herbicide residues are adsorbed tightly to soil particles with time, consequently the extraction technique is inadequate for detecting the residues. In contrast, the bound residues of herbicide can be released from the soil matrix during plant growth and thus the bioassay technique is more sensitive technique than HPLC and can detect more residues (Paul et al., 2009). The major advantage of the bioassay over the HPLC is, its relative simplicity. The HPLC assay is moderately labor-intensive and requires expensive equipment whereas the bioassay is easy to perform and requires no special equipment. According to Ranft et al. (2010), the chemical extraction measures the concentration of herbicide, but not the rate influencing the crop, while the bioassay method measures amount of herbicide available to the plant, but not unavailable active herbicide in the soil.

Our study highlighted that the estimated half-life of herbicide by HPLC was lower than that of bioassay (*Table 5*). At the recommended rate of application (26.6 g a.i. ha^{-1}), estimated half-life of herbicide by bioassay and HPLC were 20.31 and 17.28 days in 2014 and 21.78 and 19.42 days in 2015, respectively. In a study by Paul et al. (2009), the half-lives of metsulfuron-methyl by HPLC and bioassay were found to be 6.3–7.8 and 17.5 days, respectively. The ability of a soil residual herbicide to have a phytotoxic effect on a sensitive crop depends on the half-life of the herbicide being used. The half-life of herbicides in soil varies with the chemical structure and soil conditions that affect degradation. According to Sarmah et al. (1999), the half-life of a sulfonylurea herbicide in soil can vary significantly depending on pH, temperature, moisture, texture and organic matter content of the soil (Blacklow and Pheloung, 1991; Sarmah et al., 1999). Sulfosulfuron, with a field half-life of 14 to 75 days, is often one of the more persistent herbicides (Vencill, 2002).

According to the bioassay and HPLC results, at two rates of application (26.6 and 53.2 g a.i. ha^{-1}), Sulfosulfuron residues decreased with time in 2014 and 2015. This may be due to various causes. Once a herbicide is applied, there are eight possible routes that may affect its dissipation: 1. adsorption to clay and organic matter, 2. leaching with the downward percolation of water, 3. volatilization to the atmosphere, 4.uptake by plants and/or soil organisms, 5. movement with runoff water or eroded soil, 6. microbial degradation, 7. chemical degradation, and 8. photolysis (McEwen and Stephenson, 1979). These processes are affected by the adsorption of a herbicide to soil colloids and soil organic matter. The greater the adsorption of a herbicide to a particular soil, the less the losses from leaching and volatilization (Smith, 1982). Depending on the chemical nature of the herbicide the importance of each of these routes can be quite different. One of the properties of some ALS inhibiting herbicides such as Sulfosulfuron, is soil residual activity that can result in weed control throughout the growing season. However, this characteristic can also cause crop damage and an economic loss due to a phytotoxic effect on sensitive rotational crops (O'Sullivan et al., 1998). The degree to

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which a residual herbicide can persist and cause damage is influenced by three factors including soil properties, environmental conditions and landscape position (Moyer and Hamman, 2001; Schoenau et al., 2005).

According to the bioassay and HPLC results, at two rates of application, Sulfosulfuron had different soil persistence in 2014 and 2015. This may be due to various environmental conditions. The environment has a large influence on herbicide residue persistence. Many herbicides that have residual activity are degraded in soil by hydrolysis and/or microbial degradation (Beckie and McKercher, 1989; Vencill, 2002). The relative importance of microbial degradation as compared to chemical hydrolysis is dependant on many factors. Joshi et al. (1985) found that a sulfonylurea herbicide degraded faster in acidic soils because both forms of degradation took place. In alkaline soils however, microbial degradation was the primary source of degradation, resulting in a slower rate of dissipation. Temperature and soil moisture levels have a significant effect on soil microbial populations and activity. Beckie and McKercher (1989) found that lower temperatures and drier soils resulted in the ability to detect herbicide residues with a bioassay for a longer time period after application. Experimental sites, which received a higher level of precipitation, had lower amounts of phytotoxic residues of sulfonylurea herbicides present the year after application (Shinn et al., 1998). Hill et al. (1998) found that yearly precipitation levels had a significant effect on the persistence of quinclorac, with drier conditions increasing the soil residual half-life of the herbicide. This suggests that in years of lower than average growing season temperatures and/or lower precipitation, residual herbicides may persist longer in the soil. This can have negative effects on sensitive rotational crop species, resulting in reduced yield and/or later maturing crops.

Conclusion

In this study, the reliability and accuracy of HPLC and bioassay methods were evaluated to detect sulfosulfuron residues at macro quantitative levels. Results revealed that the bioassay was more sensitive than HPLC for the determination of sulfosulfuron residues. According to the bioassay experiment, root parameter was more sensitive to sulfosulfuron as compared with the shoot. Root length inhibition bioassay was a useful tool to detect small amounts of sulfosulfuron residues in the soil. So the residues of sulfosulfuron herbicide were detected up to 60 and 90 days after application by HPLC and bioassay techniques, respectively. These findings can be applicable for agriculture since knowledge on the degradation rates of herbicides usage, and their possible effects on sensitive rotational crops can help for making effective management decisions. Further studies need to be carried out for a better understanding of the sulfosulfuron behavior in different soil and meteorological conditions.

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SEASONAL POLLUTANT REMOVAL BY *LACTUCA SATIVA*, *MEDICAGO SATIVA* AND *PHRAGMITES AUSTRALIS* IN CONSTRUCTED WETLANDS

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Abstract. The main objective of this study was to compare the removal efficiency of nutrients using *Lactuca sativa, Medicago sativa* and *Phragmites australis* in subsurface flow constructed wetlands with horizontal flow. In order to test water quality, fabricated reactors designed and the plants cultivated in the soil while their root were inside the wastewater. A long time study carried out from spring till end of autumn (9 months) in order to evaluate the difference in removal rate based on the seasonal changes. The highest removal rate was during summer which followed by spring and autumn. Thus, the effect of plants on the removal efficiency of organic matter (COD, BOD), TSS and nutrient (P and TN) appeared to be dependent on the seasonal growth. *Phragmites australis* the most sensitive species in order the removal of nutrient from wastewater.
Keywords: *subsurface flow constructed wetland, nutrient removal, wastewater treatment; Lactuca sativa, Medicago sativa, Phragmites australis*

Introduction

Today, constructed wetlands are recognized as a reliable wastewater treatment technology and they represent a suitable solution for the treatment of many types of wastewater. Constructed wetlands have been used extensively across the world for treating wastewater due to its low cost and efficiency in removal of pollutants (Vymazal, 2010). Wastewater treatment in wetland occurred by a process named phytoremediation that is a moderately late innovation and is seen as practical, proficient, novel, eco-friendly technology, still in its initial improvement stages and full scale applications are still constrained (Rezania et al., 2015). Wide range of wastewaters such as municipal, industrial, agricultural, and storm water can be remediated in constructed wetland (CWs) (Pedescoll et al., 2015; Qasaimeh et al., 2015). In addition, CWs is a flexible and effective method for treating and reusing wastewater with less greenhouse gas emission (Chen et al., 2011).

Generally, the major functions of plants in CWs is to create conditions suitable for removal of pollutants and the direct role of plants is limited to uptake of nutrients and heavy metals (Marchand et al., 2010). Brisson and Chazarenc (2009) showed that some environmental factors like temperature, pH, solar radiation and water salinity can influence plant growth and its performance in phytoremediation. The importance of

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these parameters are related to size, weight and growth rate of aquatic plants (Lissy and Madhu, 2010).

Based on Tanner (2001), wetland plants provide only small improvements in biological oxygen demand (BOD), chemical oxygen demand (COD) and faecal bacterial indicator removal but provide measurable enhancement of nutrient removal, mainly by promoting transformations to gaseous forms and sequestration in accumulating organic matter. As extensively reviewed by Engloner (Engloner, 2009), considering the worldwide distribution, Phragmites dominated wetlands may have a considerable effect on climate change. Because of this issue, the significance of common reed in scientific research will be on-going in the future studies. *Phragmites australis* is the most often used plant in CWs although a large diversity of species can be used and the genus *Typha* and *Scirpus* are commonly used (Vymazal, 2013). As described by Engloner (2004), water availability is an important factor in shoot which is longer in wetter than drier habitats (with maximum water depths 40-0 and -30 cm, respectively).

In the Czech Republic, horizontal subsurface flow CWs are mostly planted with *Phragmites australis* (Common reed) or *Phalaris arundinacea* (Reed canarygrass) or with a combination of these two species. Some factors like: (1) excellent germination from the seeds, (2) easy planting, (3) fast growth, creating full cover of the surface during the first growing season if planted in spring, and (4) provision of good insulation during the winter (Vymazal, 2013). *P. australis* aboveground biomass in HF CWs varies widely between the values < 1000 g m -2 to more than 11,000 (g/m-2) on dry matter basis and growing in natural stands also varies greatly within the similar range as in constructed wetlands (Vymazal and Březinová, 2016). As the nitrogen and phosphorus are essential elements for plants and living beings, meanwhile domestic and industrial wastewater which is not fully treated can be among the most important factors that threatens the water quality (Chang et al., 2006). There has been a growing evidence that constructed wetlands with emergent vegetation are more efficient as compared to unplanted filters and that some species are more efficient than the other ones (Zhang et al., 2010).

The objective of this study was to determine the effect of nutrient removal by *Medicago sativa*, *Lactuca sativa*, *Phragmites australis* and mixture of (*Medicago sativa* and *Lactuca sativa*) while they grew in municipal wastewater as the first treatment system. Also, the removal efficiency of these plants during 9 months experiments was compared.

Experiment

Pilot experimental set-up

A pilot experimental system was established in Ekbatan wastewater treatment plan and the study was carried out from April 2015 to December 2015. In Iran, the spring begin on April while the summer and autumn begin on July and October, respectively. The system was comprised of five horizontal subsurface flow CW units in parallel with a different plant species each: CW1 (unplanted), CW2 (*Medicago sativa*) CW3 (*Lactuca sativa*), CW4 (*Phragmites australis*) and CW5 (Mixture of *Medicago sativa* and *Lactuca sativa*) as shown in *Figure 1*.

Figure 1. *Plant cultivation in separate reactors*

Each CW unit has length of 1.15, width of 0.75 and depth of 0.6 meters. Inside the reactor was completed with stones of various diameters as larger stones (15-32 mm) were placed at the bottom, medium (9.5-15 mm) and small (6-9 mm) placed at the middle and the top. The wastewater was entered with a flow rate of (0.093 L/day) to the system continually. The length of *M. sativa* root was inside the sand 10 cm while it was 6 cm for *L. sativa* is 6 cm (in a radius way). In the case of *P. australis*, the bushy root reached to the bottom of the soil. The overall design of pilot study is shown in *Figure 2.*

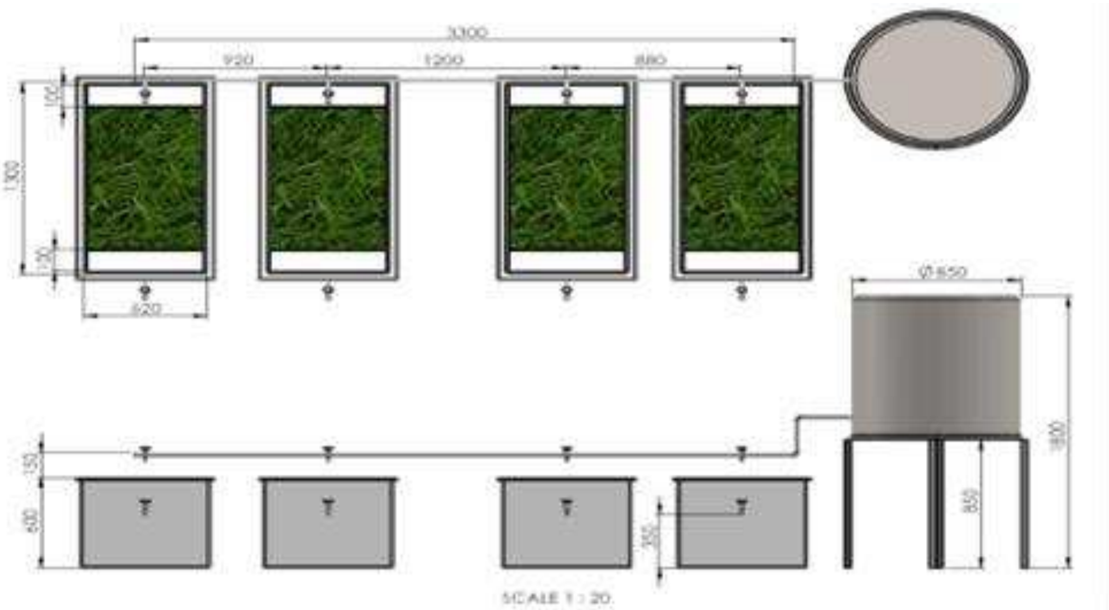

Figure 2. *Design of horizontal subsurface flow constructed wetland in the study*

Water sampling and analysis

The influent comes from raw wastewater as first treatment system which located at Ekbatan township in Tehran. The samples were collected from influent and effluent of each CWs daily for the analyses. The sampling last for 10 days of each month (standard deviation) and each test was conducted in triplicates. All the parameters were investigated in accordance to APHA (2005) BOD, COD and TSS were measured by

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following methods: BOD_5 determined using 5-Day BOD test method, COD by colorimetric method (5220-D) and TSS by using Method No. 2540D. The total P was measured using the vanado-molybdate colorimetric method by measuring the absorbance at a wavelength of 420 nm (Fiske and Subbarow, 1925). Kjeldahl nitrogen was measured using APHA (2005).

Results and discussion

The removal efficiency of each plant in CWs during 9 months in different seasons was investigated and discussed as shows in *Table 1*.

Table 1. *Removal efficiency based on different CWs*

CWs	**Plant species**	**Removal efficiency**		
		Spring	Summer	Autumn
1	*Medicago Sativa*	low	high	moderate
2	*Lactuca sativa*	moderate	high	moderate
3	*Phragmites Australis*	moderate	high	moderate
4	*Medicago Sativa & Lactuca sativa*	moderate	high	moderate

Medicago sativa

As shown in *Figure 3* during the first months of plant operation, COD decreased gradually which the lowest was 385 on 5 month in the summer. It showed the linear and stable reduction till end of the experiment. The reduction was around 50% using *Medicago sativa* for the treatment of municipal wastewater. Similarly, BOD showed same reduction while after month 5 (35%), small increases observed which may related to biomass growth and seasonal changes. In regard to nutrient removal like P and TN, the reduction occurred till 4 month (summer). In summer, the amount of P and TN increased slightly while was fluctuated for TN. During autumn, P values in water decreased proportionally while TN showed lesser reduction rate by 25%. In regard to TSS, it increased sharply till from spring till end of summer (6 months) which the highest was by 120% on 6 months. Then, it decrease during autumn till end of experiment.

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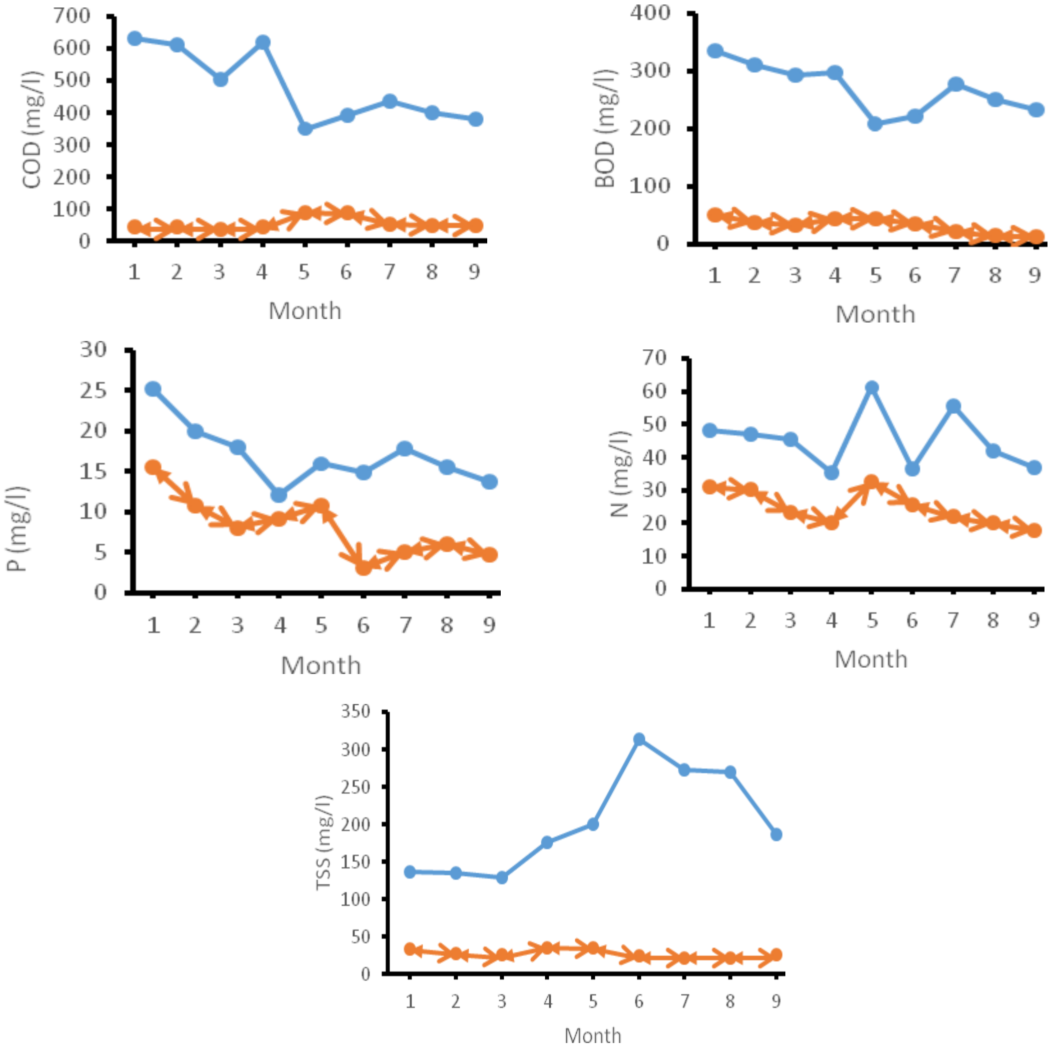

Figure 3. *Nutrient removal by M. sativa*

Lactuca sativa

There is no studies which used *Lactuca sativa* for wastewater treatment. This plant reduced COD by 40% during first five months of the experiment. Then, COD increased during autumn till end of the experiment. In regard to BOD, similar reduction occurred which was highest by 35% on middle of summer after 5 months of the experiment. *L. sativa* had similar removal reduction pattern for T and TN which reduced after 4 months of the experiment. The highest removal was 60% and 30% for P and TN, respectively. By using *Lactuca sativa*, TSS was increased till end of summer by 100% and then decreased till end of autumn (*Figure 4*).

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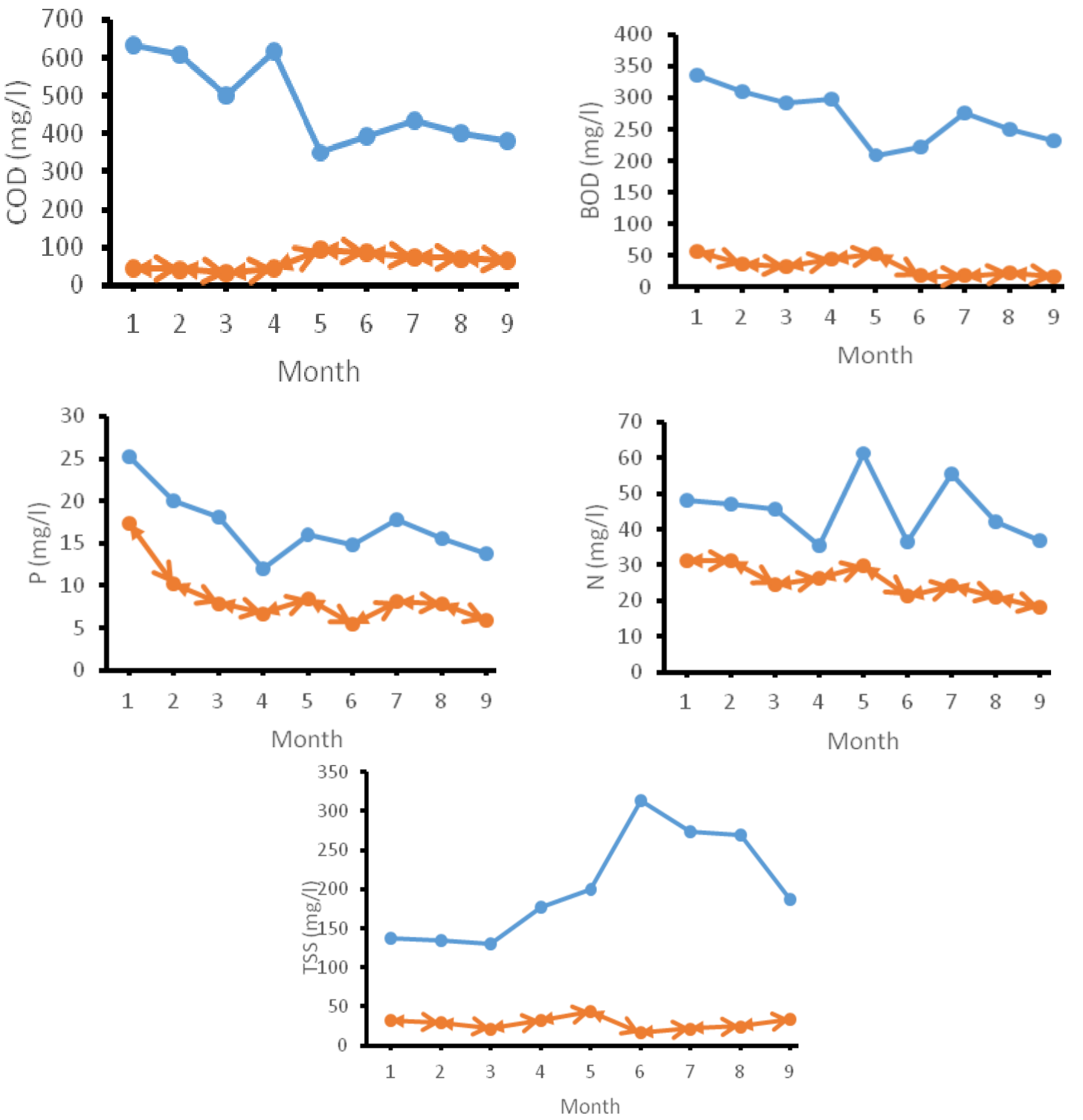

Figure 4. *Nutrient removal by L. sativa*

Phragmites australis

Many studies showed the efficiency of *P. australis* in removal of nutrient from different types of wastewater. This plant was effective in removal of BOD and COD over the five years (Březinová and Vymazal, 2014). Although, Carballeira et al. (2016) found that the high increases in TSS by using *P. australis*. In this study, *P. australis* removed COD and BOD by 40% and 30% till end of summer, respectively. Similarly, P and TN removed significantly till starting of summer by 65% and 30% respectively. Fuchs et al. (2011) found lesser removal rate of TN and P using *Phragmites australis* which was as 35.6 % and 31% respectively. As indicated by Carballeira et al. (2016), *P. australis* had an intermediate biomass production rate and an increased nitrogen removal rate by approximately 30% in comparison with the control. As shown in *Figure 5*, TSS increased till end of summer and was decreased during autumn proportionally.

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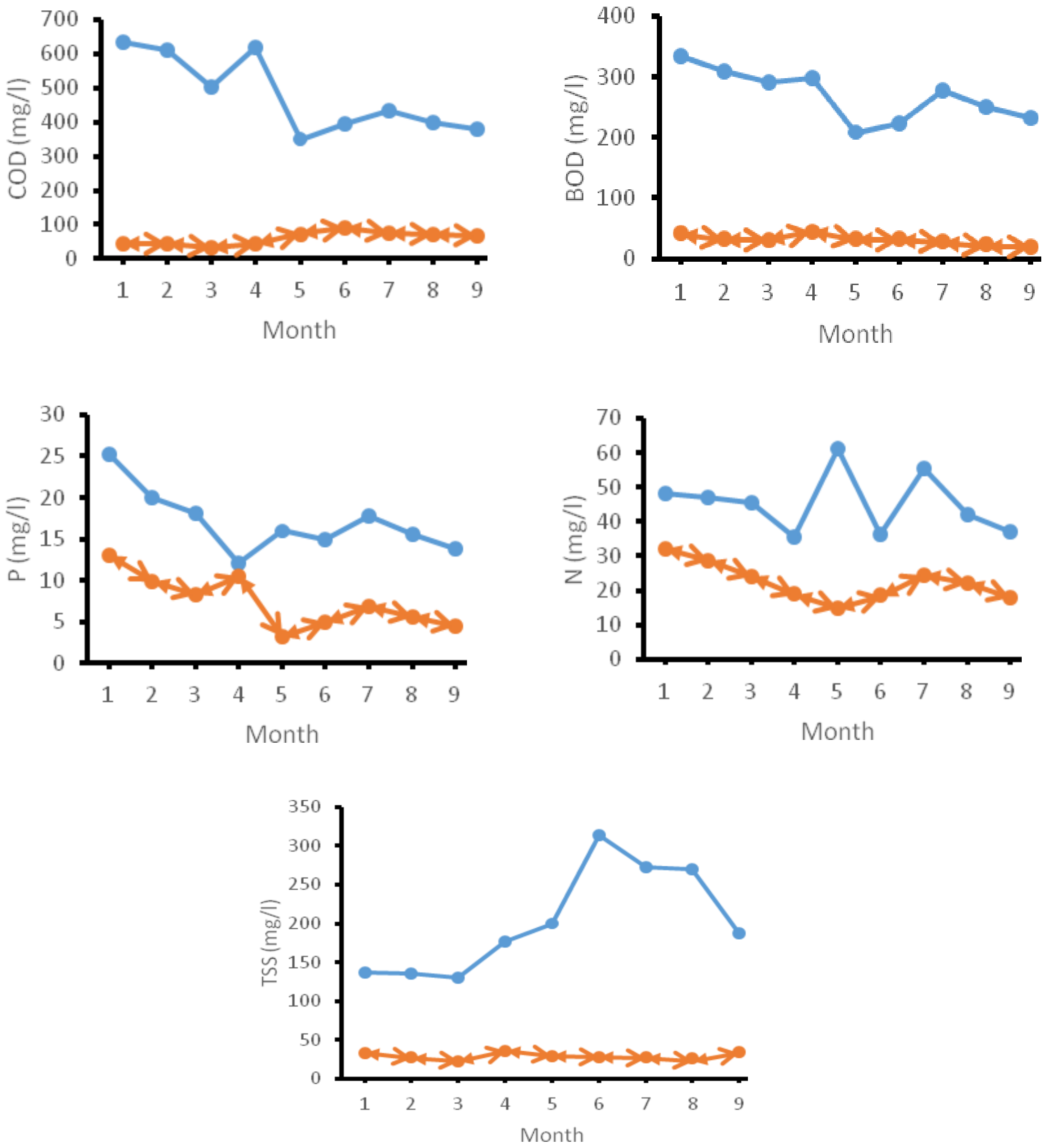

Figure 5. *Nutrient removal by P. Australis*

Mix of L. sativa and M. sativa

As shown in *Figure 6*, in the CWs contains mixture of *L. sativa* and *M. sativa*, similar observation of reduction was observed. The obtained results showed in was inconsistence with reduction of these plants when they culture in CWs alone. Most of the reduction occurred during summer while it was lesser during autumn. Highest removal of most of the parameters was in summer which followed by autumn and the lowest was in spring (*Figure 6*). It shows that the duration and season are the important factors which influenced removal efficiency.

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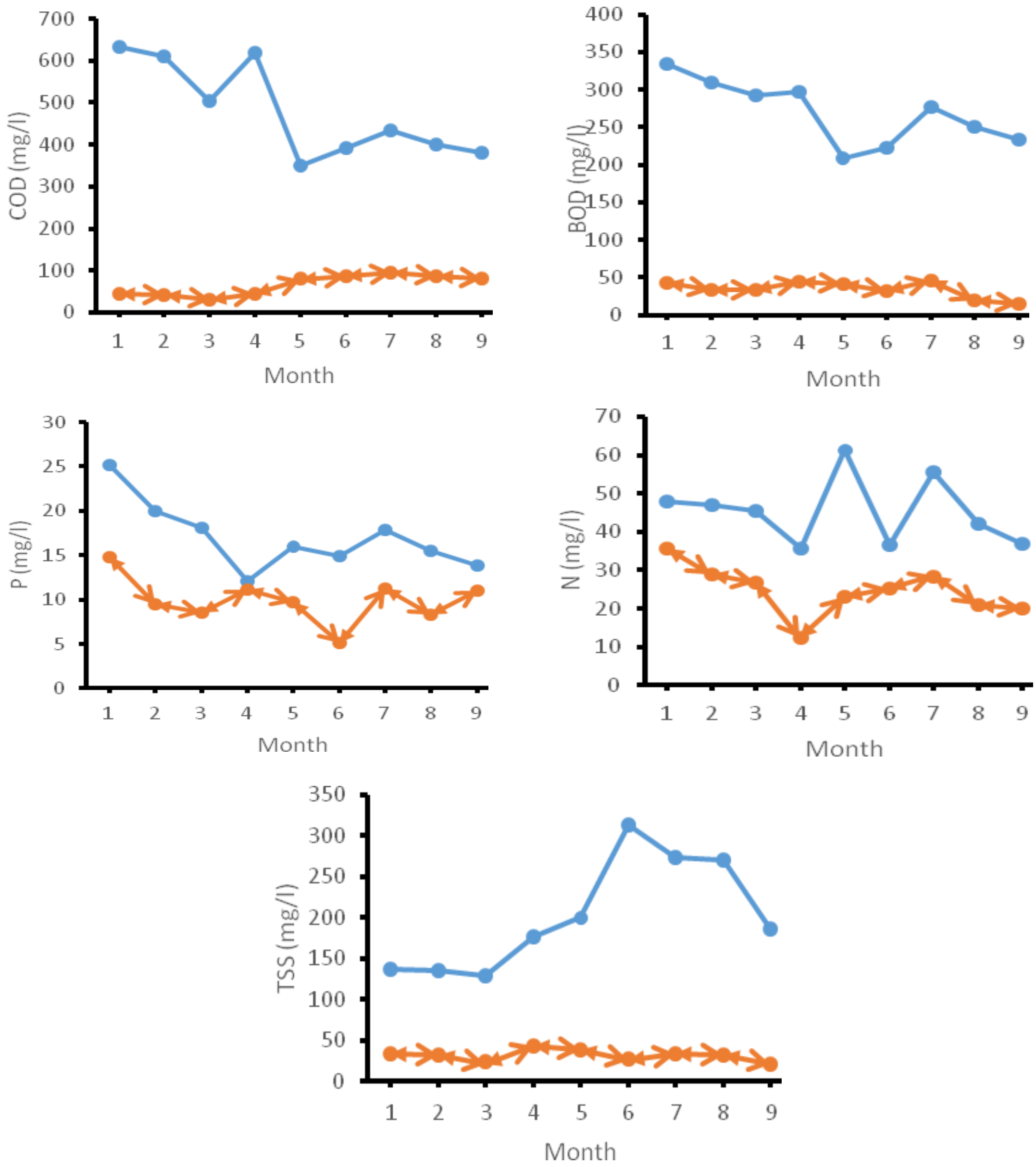

Figure 6. *Nutrient removal by mix of L. sativa & M sativa*

Conclusion

In this study, the effectiveness of nutrient removal by *Medicago sativa*, *Lactuca sativa*, *Phragmites australis* and mixture of (*Medicago sativa* and *Lactuca sativa*) in farictaed CWs was investigated. Different parameters like COD, BOD, TSS, P and TN measured during 9 months treatment system of municipal wastewater. Similar reduction pattern was observed during experiment while the highest reduction occurred during summer which followed by spring and autumn, respectively. In terms of removal efficiency in CWs, the highest reduction obtained using *Phragmites australis* which followed by (*Medicago sativa* and *Lactuca sativa*), *Medicago sativa* and *Lactuca sativa*. The average of removal rate was for COD (40-65%), BOD (30-50%), TSS (100-120% increase), P (40-60%) and TN (25-35%). This study proved that the horizontal

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subsurface flow CWs are the effective method for wastewater treatment for initial and secondary treatment.

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Conflict of Interest. The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest.

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PRODUCTION POTENTIAL PREDICTION FOR WHEAT, BARLEY AND MAIZE BASED ON SOIL CHARACTERISTICS USING ARTIFICIAL NEURAL NETWORKS IN VARAMIN REGION, IRAN

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Abstract. Due to increasing demands for agricultural products and the problems to generate new data, using proper models to predict the performance of agricultural products seems necessary. The objective of this study was to assess the ability of artificial neural networks (ANN) for yield prediction of wheat, barley and maize to determine the most important soil properties for land production potential in Varamin region, Iran. In this study, potential production was calculated using the AEZ model. ANN model inputs were soil characteristics, including the percentage of calcium carbonate, coarse fragments, gypsum, clay, silt and sand, electrical conductivity, sodium absorption ratio and pH. ANN model output was production potential. Mean absolute error, root means square error and coefficient of determination criteria were used to evaluate the performance of the ANN. The obtained results showed that the ANN models with two hidden layers provided the most accurate prediction of production potential. Mean land production potentials for wheat, barley and maize, using ANN models were predicted respectively, as 7417.73, 6810.41 and 8922.48 kg/ha. Sensitivity analysis of models showed that wheat production potential has the most dependence on sodium absorption ratio and clay percent. Sand and coarse fragments rates were the most important parameters for predicting the barley production potential. The sodium absorption ratio and electrical conductivity were determined as the relevant and effective soil properties on maize production potential. Also, the optimum model for maize production potential prediction has less accuracy than wheat and barley. The reason of this may be differences in the environmental compatibility of various products to adverse conditions.
Keywords: *FAO method, sensitivity analysis, land production potential*, *gross biomass production, climatic and plant parameters*

Introduction

Crop yield prediction has an important role in agricultural policies such as specification of the crop price. Nowadays, using suitable models to predict the performance of agricultural products is vital, since demands for agricultural products are increasing and water and land resources are limited. Crop yield is a function of several plant agents, climatic conditions, and soil and water management (Qian et al., 2009). Therefore, the calculation of crop yield and its related indicators follow up the complex nonlinear relationships that are modeled difficulty.

In recent years, crop growth models have become increasingly important as major components of agriculture related decision support systems. Crop growth and yield models are based on a combination of soil, crop and climatic variables. Crop models and decision tools are increasingly used in the agricultural field to improve production efficiency. The combination of advance technology and agriculture to improve the

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production of crop yield is becoming more interesting recently. Due to the rapid development of new higher technology, crop models and predictive tools might be expected to become a crucial element of precision agriculture (Shearer et al., 2000).

Land production potential (LPP) estimation is considered as a prerequisite for land use planning. LPP is determined based on land characteristics for specific land use for increment of production per surface area unit. In this regard, first, radiation thermal production potential (RTPP) is calculated, using different models such as FAO model. This potential is a genetical one which is not affected by water, soil and management limitations. If soil limitations are exerted in the radiation thermal production potential, LPP is resulted.

Some adaptive and non-parametric models have been recently introduced in environmental science for predictive purposes. ANN models are a powerful empirical modeling approach and yet relatively simple compared with mechanistic models. (Bishop, 1995), the method is gaining popularity for research areas where there is little or incomplete understanding of the problem to be solved, but where training data are available. The ANN can be used to develop empirically based agronomic models. The ANN structure is based on the human brain's biological neural processes. Interrelationships of correlated variables that symbolically represent the interconnected processing neurons or nodes of the human brain are used to develop models. ANN models find relationships by observing a large number of input and output examples to develop a formula that can be used for predictions (Pachepsky et al., 1996).

Safa et al. (2015) showed that the ANN model can predict wheat production based on farm conditions (wheat area and irrigation frequency), machinery condition and farm inputs (N and fungicides consumption) in Canterbury, New Zealand, with an error margin of ±9%. Drummond et al. (2003) evaluated the predictive ability of representative linear, nonlinear, and neural network techniques on a multiple site–year data set of grain yield and site and soil characteristics. The results showed that significant over fitting had occurred and indicated that a much larger number of climatologically unique site–years would be required in this type of analysis. Kaul et al. (2005) reported that ANN models consistently produced more accurate yield predictions than regression models for corn and soybean yield prediction under typical Maryland climatic conditions. Norouzi et al. (2010) predicted the biomass, grain yield, and grain protein in wheat grown in hilly regions using an ANN model under rainfed conditions in the semiarid and hilly regions of western Iran. The results indicated that the ANN models could explain 89–95% of the total variability in wheat biomass, grain yield, and grain protein content. Bagheri et al. (2012) determined the least important computer input parameters which affecting the silage maize yield using ANN. The results showed that adding the solar radiation and average relative humidity to the input parameter cause reduction in MSE and increasing the accuracy of the model in the process network training. Kitchen et al. (2003) and Safa et al. (2003) suggested that the feed forward back propagation neural network is the most commonly used neural network, which can approximate any function with arbitrary precision. The objective of this study was to assess the ability of artificial neural networks (ANN) for wheat, barley and maize production potential prediction and determination of the most important soil properties affecting land production potential in Varamin area, Tehran province, Iran.

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Materials and methods

Study area and data compilation

The study area with an approximate area of 2000 hectares is located between latitude 35° 20′ and 35° 24′ N and longitude 54° 38′ and 54° 42′ E in the Varamin area, Tehran province, Iran. The area has a mean annual rainfall of 170 mm and mean annual temperature of 17.4°C with a mean altitude of 972 m a.s.l. The ground water table depth is more than 10 m. Based on U.S. Soil Taxonomy (Soil Survey Staff, 2014), the soil moisture and temperature regimes of the area are aridic and thermic, respectively.

Some of the plant characteristics such as leaf area index and harvest index were measured in the laboratory. The maximum leaf photosynthesis rate was estimated from the graph according to the crop group (Sys et al., 1991). Required climatic data for land suitability evaluation were obtained from Varamin Synoptic Meteorological Station for a 20 years period (1994–2014).

In this research based on semi-detailed soil survey, a regular grid sampling method was designed; consequently 50 soil profiles with 600 m intervals were investigated. Soil samples were collected from different horizons of the profiles. Prepared samples were subsequently analyzed for required soil properties in land suitability evaluation (Sys et al., 1993) using standard methods (Soil Survey Staff, 1996). For determining the mean values of the soil physical and chemical properties for the upper 1 m of the soil depth, the profile was subdivided into 4 equal sections and weighting factors of 1.75, 1.25, 0.75, and 0.25 were attributed for each section, respectively (Sys et al., 1991).

The land characteristics, i.e., climatic data and soil properties, with studied plant requirement tables presented by Sys et al. (1993), were matched. Consequently, the square root formula was used to calculate the soil index (SI). Soil index reflects the soil, topography and drainage limitations for irrigated farming. The relevant equation is as follows:

$$SI = R_{min} \times \sqrt{\frac{A}{100} \times \frac{B}{100} \times \ldots} \quad \text{(Eq.1)}$$

where, SI, is the specified soil index; A, B, etc., are different ratings for each soil characteristic and R_{min} is the minimum rank or value (Sys et al., 1991).

To calculate the radiation-thermal production potential of wheat, barley and maize based on FAO model (Sys et al., 1991), the following were measured or calculated:

- Respiration coefficient was calculated as follows:

$$Ct = C30(0.044 + 0.0019t + 0.001t^2) \quad \text{(Eq.2)}$$

where, Ct, respiration coefficient; C30 of 0.0108 for non-legumes and t, mean daily temperature of the growing cycle (°C).

- Maximum gross biomass production ratio was calculated according to:

$$bgm = f \times bo(1 + 0.002\,y) + (1 - f) \times bc(1 + 0.005\,y) \quad \text{(Eq.3)}$$

$$y = \left|(Pm - 20) \times 5\right| \quad \text{(Eq.4)}$$

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where, bgm, maximum gross biomass production rate (kg CH_2O/ha.hr); f, fraction of the daytime that the sky is overcast; bo, maximum gross biomass production on overcast days (kg CH_2O/ha.day); bc, maximum gross biomass production on clear days (kg CH_2O/ha.day) and Pm, maximum leaf photosynthesis rate (kg CH_2O/ha.hr).

- Radiation-thermal production potential was calculated as follows:

$$Y = (0.36 \times bgm \times KLAI \times Hi) / \left[1/_L \times 0.25Ct \right] \quad \text{(Eq.5)}$$

where, Y, radiation-thermal production potential (kg/ha); bgm, maximum gross biomass production rate (kg CH_2O/ha.day); KLAI, leaf area index at maximum growth rate; Hi, harvest index; L, growth cycle (day) and Ct, respiration coefficient.

Finally, LPP was calculated according to:

$$LPP = Y \times SI \quad \text{(Eq.6)}$$

where, LPP, the land production potential (kg/ha); Y, radiation-thermal production potential (kg/ha) and SI, the soil index.

Land and soil characteristics were included topography, wetness and drainage conditions, soil texture and structure, the percentage of coarse fragments, equivalent $CaCO_3$ percent, gypsum percent, soil depth, electrical conductivity (EC), sodium absorption ratio (SAR) and pH. The growing period was determined based on climatic data (i.e., rainfall, temperature, relative humidity, hours of bright sunshine and wind speed) using graphical method. To calculate the evapotranspiration, CropWat software was used.

Artificial neural network model development

In this paper, the feed forward back propagation neural network as the most commonly used neural network architecture was applied. The first term, “feed forward” describes how this neural network processes and recalls patterns. “Back-propagation” is a form of supervised learning where the error rate is sent back through the network to alter the weights to improve prediction and decrease error. Supervised learning, which applies known outputs to train the ANN, is more commonly used than unsupervised learning (Wieland and Mirsche, 2008).

Since, the climatic characteristics and topographic conditions were entirely equal within the study area; they were not considered as the ANN inputs. ANNs can identify and learn correlated patterns between input data sets and corresponding target values through training. The trained network is then tested with a separate data set with its output information omitted. Training data sets were used to develop models including field-specific: equivalent $CaCO_3$ percent, percentage of coarse fragments, gypsum, clay silt and sand percent, EC, SAR and pH as inputs with associated land production potential as output. The training and testing process have been carried out for around 80% and 20% of the sample data set respectively in each of the cases under study.

Since, the sigmoid threshold function was used in the hidden layer and its output values are between 0 and 1, therefore the data sets were normalized using the following equation (Montazar et al., 2009):

$$X_n = 0.5\left(\frac{X_o - X_{ave}}{X_{max} - X_{min}}\right) + 0.5 \quad \text{(Eq.7)}$$

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where X_n, the normalized data; X_o, the input data; X_{ave}, the average of data, X_{min} and X_{max}, the minimum and maximum data value, respectively.

Training neural network models was founded on the basis of trial and error. Adjustment of ANN parameters were included the number of hidden nodes and hidden layers, learning rate, training function type and training tolerance. So that, the optimum neural network architecture was obtained from changing the mentioned parameters. For each step, multiple linear regression models between the actual and predicted yields were applied. The coefficient of determination (R^2) and root mean square error (RMSE) were calculated to find the optimum neural network architecture. The different threshold functions such as logarithmic sigmoid function, linear and hyperbolic tangent functions were used to improve neural network performance. Finally, the best neural network architecture was obtained for prediction of wheat, barley and maize land production potential. The performance of developed models was evaluated using by mean absolute error (MAE), RMES and R^2 (Tang et al., 1997). The ANN models were developed in MATLAB 10.1 software.

The sensitivity analysis must be conducted to detect the robustness of every ANN model because different choices of function and parameters in ANN models would influence the performance of simulation (Zhang et al., 2007). In this research, sensitivity analysis was done using Statfsoft method (StatSoft, 2004). The sensitivity coefficient values of the inputs obtained by dividing the total network error in the absence of one variable on the total network error in the presence of all the variables. According to this, if the sensitivity coefficient value of variable is more than 1, the variable has a great impression in the variability of the components of yield (Norouzi, 2010).

Results and discussion

Required plant and climatic characteristics for calculation of wheat, barley and maize radiation-thermal production potential were presented in *Table 1*. Regarding to FAO model, the radiation-thermal production potential of wheat, barley and maize were obtained 7417.73, 6810.41, and 8922.48 (kg/ha), respectively. Of course, it is impossible to achieve such production because anyway, there are soil and management limiting factors in irrigated farming. In other words, the difference between the LPP and farmer yield is due to these limitations. *Table 2* shows summary statistics of the studied soil attributes used in the training and testing process. The studied soils have a wide range of EC, SAR and particle size distribution (*Table 2*).

The designed ANN models

In this study, one and two hidden layer structure and one to 14 numbers of neurons in hidden layers was applied in network training. Eight optimized ANN models for wheat and maize and seven optimized models for barley production potential predication were achieved. *Table 3* shows the characteristics of ANN models. The efficiency of models for estimation of LPP was compared with R^2, RMSE and MAE statistics (*Table 4*).

Table 1. *Required crop and climatic parameters for calculation of radiation-thermal production potential for wheat, barley and maize*

Parameters	**Wheat**	**Barley**	**Maize**
Crop group	C3, Group 1	C3, Group 1	C4, Group IV
Mean temperature of the growing cycle (°C)	22.0	20.13	31.53
Maximum leaf photosynthesis rate (kg CH_2O/ha.hr)	20	20	65
Leaf area index ($m^2.m^{-2}$)	4.5	4.5	4.0
Harvest index	0.45	0.40	0.35
Mean of bo (kg CH_2O/ha.hr)	204.24	192.01	243.83
Mean of bc (kg CH_2O/ha.hr)	394.44	373.90	461.17
f	0.25	0.27	0.13
Ct	5.2×10^{-3}	4.4×10^{-3}	1.1×10^{-2}
Radiation-thermal production potential (kg/ha)	7417.73	6810.41	8922.48

Table 2. *Summary statistics of data sets in testing and training steps neural networks*

Soil characteristics	**Training step**				**Testing step**			
	Minimum	Maximum	Mean	Cv[a] (%)	Minimum	Maximum	Mean	Cv (%)
EC (dS/m)	0.4	14.8	4.1	90.5	1.2	14.3	4.5	93.5
pH	7.5	8.7	8.2	3.8	8.2	8.8	8.2	4.1
SAR	0.2	35.2	9.2	95.5	3.7	26.4	8.3	96.6
$CaCO_3$ (%)	5.7	14.8	9.1	2.3	6.8	11.7	8.3	2.7
Coarse fragments (%)	0.0	49.0	23.6	69.3	26.2	42.8	24.5	61.5
Gypsum (%)	1.0	6.2	1.7	69.6	1.1	2.1	1.3	24.8
Clay (%)	0.0	16.6	6.5	79.5	0.0	13.7	5.1	100.1
Silt (%)	5.5	56.6	18.2	62.8	9.2	32.4	16.8	42.8
Sand (%)	30.3	94.5	75.3	19.9	88.0	92.5	78.1	15.2

[a] Coefficient of variation

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Table 3. *Characteristics of artificial neural networks models*

	Model	1	2	3	4	5	6	7	8
All crops	Network type	MLP[a]	MLP	MLP	MLP	MLP	MLP	MLP	MLP
	Training method	BP[b]	BP	BP	BP	BP	BP	BP	BP
	Training process	Epochs	Epochs	Epochs	Epochs	Epochs	Epochs	Epochs	Epochs
Wheat	Training function	bfg	br	gdm	scg	gdm	br	scg	bfg
	No. of hidden layers	1	1	1	1	2	2	2	2
	No. of neurons in hidden layers	12	9	8	7	5-9	6-8	9-12	7-9
	Epochs	16	16	16	16	16	16	16	16
	Momentum	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Barley	Training function	scg	scg	gdm	bfg	gdm	scg	scg	-
	No. of hidden layers	1	1	1	1	2	2	2	-
	No. of neurons in hidden layers	13	10	8	5	9-12	8-10	5-11	-
	Epochs	16	16	16	16	16	16	16	-
	Momentum	0.6	0.6	0.6	0.6	0.6	0.6	0.6	-
Maize	Training function	bfg	br	scg	scg	scg	br	bfg	bfg
	No. of hidden layers	1	1	1	1	2	2	2	2
	No. of neurons in hidden layers	10	9	8	6	6-8	9-11	7-9	6-9
	Epochs	16	16	16	16	16	16	16	16
	Momentum	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3

[a] Multi-layer perceptron
[b] Back propagation

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Table 4. *Statistical results of tested artificial neural networks models for prediction of wheat, barley and maize production potential*

Crop	**Model**	**Testing step**		
		R^2	**RMSE**	**MAE**
Wheat	1	0.698	0.326	0.249
	2	0.743	0.401	0.344
	3	0.690	0.360	0.299
	4	0.723	0.475	0.366
	5	0.703	0.314	0.189
	6	0.682	0.193	0.251
	7	0.780	0.310	0.245
	8	0.686	0.285	0.074
Barley	1	0.809	0.105	0.216
	2	0.752	0.092	0.202
	3	0.873	0.559	0335
	4	0.827	0.449	0344
	5	0.738	0.426	0.036
	6	0.863	0.586	0.125
	7	0.892	0.299	0.235
Maize	1	0.605	0.221	0.270
	2	0.600	0.542	0.165
	3	0.707	0.499	0.399
	4	0.731	0.470	0.364
	5	0.746	0.426	0.352
	6	0.669	0.301	0.268
	7	0.728	0.653	0.194
	8	0.659	0.635	0.231

The ANN models were ranked based on the highest R^2 and the lowest RMSE and MAE for selection the best one. Numerical values were assigned to the best and worst models (1 to 8 for wheat and maize and 1 to 7 for barley). Based on the sum of these values, ANN models were ranked: the models with the lowest and the highest sum of scores had the highest and lowest worthiness, respectively. Ranking of models for selection of the optimum model was presented in *Table 5*. The results for each crop were as follows:

Wheat production potential prediction by proposed ANN model

Model No. 7 in *Table 3* as the best ANN model for estimation of wheat production potential, has 10 input layers and one output layer. The number of neurons in two hidden layers has been taken as 9 and 12. This model has the lowest sum of the scores and the highest ranking in training process (*Table 5*). *Fig. 1* shows high correlations between calculated FAO production potential and predicted ANN production potential (R^2= 0.924). High coefficient of determination reflects the ability of ANN for estimation of the wheat production potential. Also, R^2 of 0.780, RMSE of 0.310 and MAE of 0.245 in training stage confirm the efficiency of the proposed ANN model

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(*Table 4*). The highest and the lowest estimated wheat production potential by the optimum model were: 5013 and 749 (kg/ha), respectively (*Table 6*).

Table 5. *Ranking of artificial neural networks for selection of optimum model*

Crop	Model	Scores			Sum. of scores	Optimum model
		R^2	RMSE	MAE		
Wheat	1	5	5	4	14	7
	2	2	7	7	16	8
	3	6	6	6	18	5
	4	3	8	8	19	6
	5	4	4	2	10	1
	6	8	1	5	14	2
	7	1	3	3	7	3
	8	7	2	1	10	4
Barley	1	5	2	4	11	7
	2	6	1	3	10	2
	3	2	6	6	14	1
	4	4	5	7	16	5
	5	7	4	1	12	6
	6	3	7	2	12	3
	7	1	3	5	9	4
Maize	1	7	1	5	13	5
	2	8	6	1	15	6
	3	4	5	8	17	1
	4	2	4	7	13	7
	5	1	3	6	10	4
	6	5	2	4	11	2
	7	3	8	2	13	8
	8	6	7	3	16	3

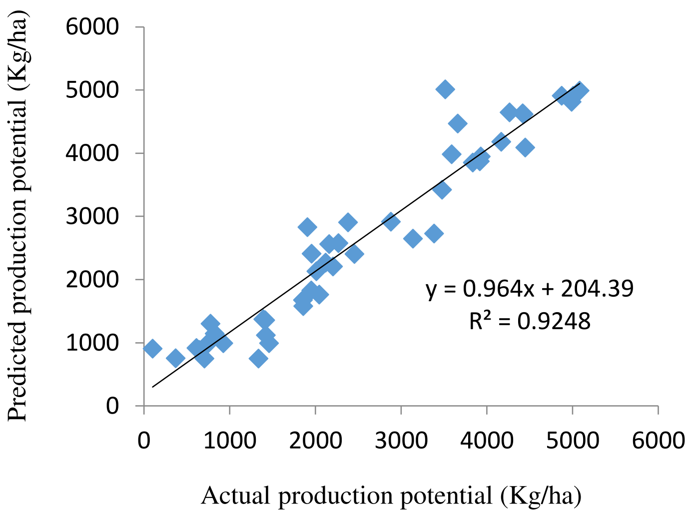

Figure 1. *Relationship between actual and predicted production potential for wheat by neural network*

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Table 6. *Summary statistics of actual and predicted production potential by artificial neural networks*

Crop	Production potential (kg/ha)	Minimum	Maximum	Mean
Wheat	Actual production potential	101	5080	2503
	Predicted production potential by ANN	749	5013	2617
Barley	Actual production potential	95	4769	2350
	Predicted production potential by ANN	252	4764	2389
Maize	Actual production potential	124	6234	3072
	Predicted production potential by ANN	667	6083	3354

According to *Table 7*, SAR and clay percent, which have the coefficient of sensitivity: 6.12 and 5.96, respectively, were as the most effective factors for wheat production potential prediction. The reason for the yield decline due to salinity has been attributed to competition between sodium and chloride ions in nutrient uptake. In saline soil, the balance in nutrient uptake was disturbed (Hosseinifard et al., 2005). In coarse soil texture, high percentage of nitrogen was leached out of the root zone. Although in fine soil texture, nitrogen leaching is more limited than sandy soils, but more nitrogen volatilization was occurred (Salehi et al., 2009).So, mineral soil particles (sand, silt and clay) as the effective factors on soil fertility are very important in crop yield. The finding results of this research also confirm it.

Table 7. *Sensitivity coefficients of used parameters in the neural network*

Removed factor	Wheat		Barley		Maize	
	Sensitivity coefficient	RMSE	Sensitivity coefficient	RMSE	Sensitivity coefficient	RMSE
-	-	0.170	-	0.241	-	0.234
EC (dS/m)	4.92	0.836	1.54	0.371	6.65	1.556
pH	2.15	0.366	2.85	0.687	1.98	0.463
SAR	6.12	1.040	1.95	0.470	7.26	1.699
$CaCO_3$ (%)	3.72	0.632	2.23	0.537	2.26	0.529
Coarse fragments (%)	3.55	0.604	4.86	1.171	2.75	0.644
Gypsum (%)	1.53	0.260	2.98	0.718	3.96	0.927
Clay (%)	5.96	1.013	3.5	0.844	4.25	0.995
Silt (%)	1.65	0.281	0.86	0.207	0.69	0.161
Sand (%)	2.96	0.503	6.45	1.554	3.12	0.730

Barley production potential prediction by proposed ANN model

Model No. 7 as the best ANN model (*Table 5*) for estimation of barley production potential, has 10 input layers and one output layer. The number of neurons in two hidden layers has been taken as 5 and 11. *Fig. 2* shows the correlation between the calculated FAO production potential and the predicted ANN production potential for

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barley (R^2 = 0.866). This model estimated the highest and the lowest barley production potential of 4764 and 252 (kg/ha), respectively (*Table 6*). The results of coefficient of determination, RMSE and MAE, which are 0.892, 0.299 and 0.235, respectively; confirmed the effectiveness of proposed model (*Table 4*).

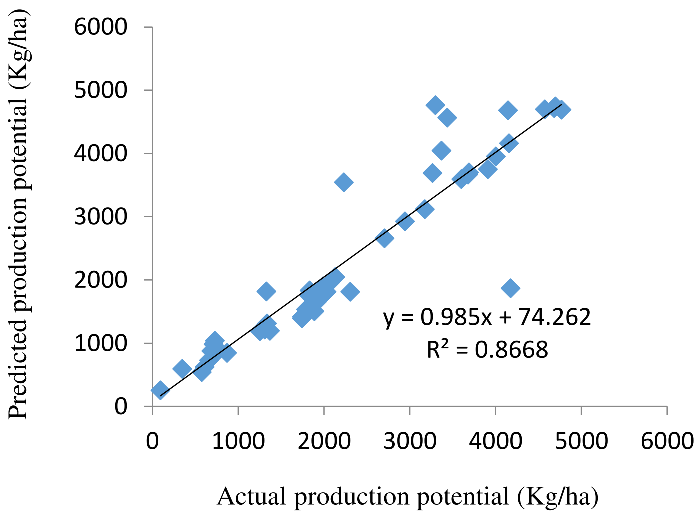

Figure 2. *Relationship between actual and predicted production potential for barley by neural network*

The results of sensitivity analysis indicate that the percentage of sand with the coefficient of sensitivity of 6.45 is the most important parameter for predicting of barley production and after that is the coarse fragment percent with the sensitivity coefficient of 4.86 (*Table 7*). Since, the studied soils with sand and coarse fragments of 75.3% and 23.6%, respectively (*Table* 2) are gravelly soils, therefore these factors are the main constraint for barley production in the study area. The reason for this could be a direct or indirect impact of these factors on the supply and maintenance of required air, water and nutrients for plant. Ranjbar et al. (2015) have reported similar findings about the influence of some soil physical properties included percentage of coarse fragments, mineral soil particles (sand, silt and clay) and weighted mean diameter of aggregates on nutrient uptake. Also, according to the growing requirements of barley (Sys et al., 1993), coarse soil textures are unsuitable for barley production.

Maize production potential prediction by proposed ANN model

Model No. 5 with two hidden layers and a network structure of 9-6-8-1 (*Table 3*) shows the highest ranking in training process (*Table 5*). *Fig. 3* shows high correlations between calculated FAO production potential and predicted ANN production potential for maize (R^2 = 0.868). The highest and the lowest estimated maize production potential by optimum model were: 6083 and 667 (kg/ha), respectively (*Table 6*). The results of coefficient of determination, RMSE and MAE, which are 0.746, 0.426 and 0.352, respectively; confirmed the effectiveness of proposed Ann model (*Table* 4). According to *Table 7*, SAR and electrical conductivity, which have the coefficient of sensitivity: 7.26 and 6.25, respectively, were the most effective factors for maize production

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potential prediction. Available information about the growing requirements of maize (Sys et al., 1993) shows the more sensitivity of maize to soil salinity and sodium content than wheat and barley. The results of studies by Khaghani et al. (2012) showed that the toxicity of chloride and sodium ions compared to chloride and magnesium ions in equal osmotic pressure led to the more reduction of maize performance.

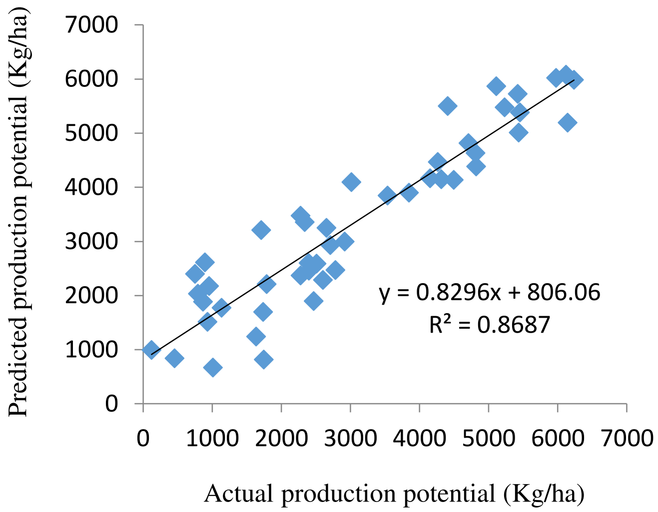

***Figure 3.** Relationship between actual and predicted production potential for maize by neural network*

RMSE, MAE and R^2 statistics of suggested models show that ANN models with two hidden layers have higher efficient than those with one hidden layer for production potential prediction of all three products (*Tables 4* and *5*). Safa et al. (2003), Kaul et al. (2005), Alvarez (2009) and Bagheri et al. (2012) showed that the efficiency of ANN model is increasing with the increment of hidden layer numbers. Although these models with two hidden layers were the most efficient model for estimation of wheat, barley and maize production potential, but the number of neurons in each hidden layer for each crop is different (*Table 3*). The reason of this can be the difference between climatic and soil requirements of each crop. Askari et al. (2009) investigated the efficiency of ANN for estimation of wheat, barley and maize yields. He found similar results in terms of the number of hidden layers and the number of neurons in the hidden layers of the optimum ANN models.

RMSE, MAE and R^2 statistics in the testing process (*Table 4*) show the higher efficiency of model for barley predicts yield (model 7) than wheat (model 7) and maize (model 5) and also the higher efficiency of model for wheat yield prediction compared to maize. The reason of this may be differences in the environmental compatibility of various products to adverse conditions. So that, barley has higher environmental compatibility compared to wheat and maize. It is also true about wheat compared to maize. Drummond et al. (2003) mentioned the more capability of ANN for yield prediction of crops with more resistance and environmental comparability.

High correlations between calculated FAO production potential and predicted ANN production potential for studied crops demonstrated that that the reliability of ANN for

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production potential prediction is acceptable in this research. This proves the high accuracy of ANN method in accordance with the nonlinear performance of crops and the influence of many factors such as climate, soil and management on it. The ability of neural network method in describing the nonlinear relation of variables for yield prediction was reported by Zare Abyaneh (2012).

Conclusions

The ANN can predict the crop production potential by land characteristics in the study area. The ANN models with two hidden layers provided the most accurate prediction of crop yield. Also, the optimum model for maize production potential has less accuracy than wheat and barley. It may be due to differences in the environmental compatibility of various crops to adverse conditions. Therefore, it can be said that the higher environmental compatibility affects the efficiency level of ANN for production potential prediction. Generally, the results of this research show that ANN method has high capability to predict the land production potential. It can be attributed to the nonlinear relation of the production potential with effective variables and the ability of neural networks in nonlinear mapping. It should be noted that using climatic and plant parameters must be considered in neural networks modeling in areas with different agricultural, ecological zones.

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A COMPARATIVE EVALUATION OF MUNICIPAL SOLID WASTE LANDFILL SITES IN NORTHERN IRAN

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Abstract. Not only in developing countries has remained land-filling the most customarily used method despite the increased attention to development of the Municipal Solid Waste (MSW) management. There is a worldwide need to find the right management of MSW and this research is developing an strategy for landfill sites in Iran. We first determine the characteristics of the ten landfill sites in western part of Mazandaran province, northern Iran, using seven different landfill site sitting approaches. After reviewing each waste deposit site, considering all the criteria in each one of the seven methods applied in the present study, the incoherence in suitability was apparent in first phase. The second phase was to address the locally preferred criteria for landfill site evaluation. The aims of the present research were to show the unsuitability of the current landfill sites in western part of Mazandaran province and therefore, addressing locally preferred criteria for landfill site evaluation in northern Iran, commonly referred to as the Caspian Landfill Criteria (CLC), using which the most suitable areas for landfill sites were formerly chosen. The most principal parts in the CLC model were the importance of the weights of the criteria and the preferred weights of them. This model with eighteen effective and native criteria provides the suitable evaluation technique for municipal landfill sites. The final aim of this project was to apply the obtained results to illustrate the lack of suitable regions for MSW landfill sites in studied area.
Keywords: *Evaluation method, Caspian Landfill Criteria, Iran, GIS, AHP*

Introduction

During the last decade improvements about how to manage the solid waste in the cities was developed. Landfills have been a quick and efficient solution but still there are many improvements to be done (Adamcová et al., 2016; Cassinari et al., 2016; Chen et al., 2015; Wong et al., 2016a, 2016b). Solid waste management is a challenging issue (Guerrero et al., 2013) as waste disposal in developing countries is one of the significant environmental challenges (Baun and Christensen, 2004; Sharholy et al., 2008). Waste generation has grown rapidly over the recent years in Iran. It has been shown that new capabilities and capacities are required to address the crisis of growing waste production in Iran, as well as new disposal centres. The location of a disposal centre is an important factor, which should be considered in construction of any new centre (Shahabi, 2008). Although, the municipal solid waste (MSW) management has been developed worldwide, it is still in a critical status in Iran (Abdoli, 2005; Yazdani et al., 2013). In most Iranian cities, landfills are not used. About less than 50% of the municipal solid waste disposal methods in Iranian cities is still confined to pile-up or other unsafe methods of disposal (Rahim et al., 2005). Open dumping is a common method of waste

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disposal in most Iranian cities (Yazdani et al., 2015a). Numerous problems are seen in the landfill sites in Iran including those in Mazandaran province. Open-air waste burning, open-pit dumping and unsafe disposal considerations are common procedures in this province which can result in irreparable damages in the environment and also on society health (Yazdani et al., 2013 and 2015a; Calvo et al., 2005; Calvo et al., 2007; Diaz et al., 2005). Some land degradations caused by landfills have been previously reported, for instance, the impacts of landfills on soil quality. It has been shown that the leachate from landfills causes the soil degradation (Hernandez et al., 1998; Raman and Narayanan, 2008; Shaylor et al., 2009). The important environmental problems affiliated with open dumps are the infiltration and ground water pollution and the subsequent contamination of the land (Kale et al., 2010; Fatta et al., 1999). The leachate from municipal solid waste (MSW) landfills is a chemical compound. Therefore, small amounts of leachate can pollute soil which can contaminate a large amount of groundwater (Nema et al., 2009; Dimitriou et al., 2008; Mor et al., 2006). For example, leachate changes the nitrate level of soil. Nitrates are easily transported with water and therefore it pollutes surface and subsurface waters (Novara et al., 2013). Uncontrolled waste incineration resulted from the degradation process is one of the other important issues in the open dumping sites (Yazdani et al., 2015b). In the present studied area, uncontrolled waste incineration is the common process in most of the 10-landfill sites. Some studies have reported the negative impacts of the fire, resulted from waste incineration, on soil quality by affecting the chemical and microbiological properties of the soil (Martínez-Murillo et al., 2016; Pereira et al., 2016) In natural ecosystems, such as existing landfill sites in the studied area, restoration of the degraded soil is achieved by returning the microbiota activity and plant community recovery. Soil development is dependent on the erosion severity, total nitrogen and pH (Yazdani et al., 2015b), since these parameters in soil texture is determined the soil typicality (Pallavicini et al., 2014).

Therefore, the attempt of the present study was to understand the quality of existent landfill sites. During the last decades multiple methods have been applied to develop more suitable municipal solid waste landfill sites. Some relevant studies have integrated GIS with MCDA (Multi Criterai Decision Making) on landfill site sitting in several procedures, some of which are mentioned below.

Alanbari et al. (2014) and Sureshkumar et al. (2017) have used Multi Criteria Decision Analysis and GIS for municipal solid waste landfill site sitting. Elahi et al. (2014) have applied Multi Criteria Decision Analysis after preparing the data and evaluating the criteria according to the geographic situation of the studied area and have overlaid the map layers with the relevant criterion in ArcGIS. This recent study finally presented 3 places in Tafresh city for municipal landfill site sitting.

Moeinaddini et al. (2010) have used spatial cluster analysis (SCA) method and weighted linear combination (WLC) to choose the proper options for MSW landfill site in Karaj city. The most preferred site was then identified by Analytical Hierarchy Process (AHP). This study has indicated that WLC was useful for identification of the criteria and AHP was useful for prioritization.

In another research carried out by Eskandari et al. (2012) a methodology based on socio-cultural and economical-ecological aspects using multi criteria evaluation integrated with GIS has been suggested to choose a proper MSW landfill site in Marvdasht city. Delgado et al. (2008) have presented three spatial analysis models (overlay analysis, Boolean logic, binary evidence) for MSW landfill site sitting.

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Furthermore, available MSW landfills are evaluated by some procedures, such as Monavari 95-2 (Monavari and Shariat, 2000; Farzaneh, 2003; Ghanbari et al., 2011), Oleckno method (Salimi et al., 2013; Monavari and Arbab, 2005), Drastic (Wang, 2007), USEPA method (Christensen, 1992) and regional and locally screening (Davami et al., 2014; Aliowsati et al., 2013).

Human beings critically influence their environment, therefore, disorder in any element of a municipal system may cause a deficiency in the entire system (Meshkini et al., 2007). Although, various waste disposal approaches have significantly been developed worldwide, land-filling as yet is the most popularly applied procedure in third world countries (Yazdani et al., 2015b; Sumathi et al., 2008). In Iran, MSW land-filling is expanded day by day because of the rapid urban population growth and the changes in consumption patterns (Davami et al., 2014; Eskandari et al., 2012).

The aims of the present research were to show the unsuitability of the current landfill sites in studied area and therefore, addressing the locally preferred criteria for landfill site evaluation in the northern Iran, commonly referred to as the Caspian Landfill Criteria (CLC), using which the most suitable areas for landfill sites were formerly chosen. The final aim of this project was to apply the obtained results to illustrate the lack of suitable regions for MSW landfill.

The landfill sites are serious issues in the western part of Mazandaran due to the geographic conditions, including the proximity of forests and the sea, high groundwater levels and high tourists in holiday seasons, this region is in a more sensitive condition than the other parts of Mazandaran province (Yazdani et al., 2013). After the evaluation of landfill sites and showing the indications of land degradation, it is essential to restore these lands. Numerous researches have been carried out on land recovery after degradation using different methods which can be used in the future. For instance, there has been a report on restoration of mine dunes with fungi species (de Suza et al., 2011). Furthermore, in another study, limestone quarries has been resorted using three approaches; tree and shrub planting, no herb layer and hyseed (Gillardeli et al., 2013). (Pallavicini et al., 2014) have emphasised that environmental factors are very important for soil quality and development. It has also been shown that to forestation on degraded land (Haigh et al., 2013). Moreover, further researches have been carried out on forest restoration as well (Quinonero et al., 2016; Davies et al., 2010). Few studies have been conducted in Iran in the land restoration field (Sadeghi et al., 2015; Fallahzadeh et al., 2015). As to the sustainable municipal solid waste management, a wise plan in the western part of Mazandaran province should be prepared and performed.

Materials and methods

Study area

The studied area (8761.5 km^2) is located in the western part of Mazandaran, Iran, which is situated in the southern coast of the Caspian Sea in northern edge of Iran. In a region consisting about 36.88 percent of the total area of this province about 20.87 percent of the population of Mazandaran province is concentrated (Iranian Statistic Centre, 2010). The 610120 inhabitants of this area generate about 181040 tons of waste per year. The elevation of the studied area varies from 27 meters below sea level to almost 4800 meters above sea level because of locating between the Alborz mountain range and the Caspian Sea. There are three distinct geomorphologic conditions with different weather conditions in the studies area;

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coastal plains and foothills with temperate and humid weather and mountains with cold weather (Mazandaran Governor, 2014).

In Iran, similarly to the other developing countries, landfilling has continued as customarily used procedure despite the increased attention to develop the MSW management (Yazdani et al., 2013; Eskandari et al., 2012; Sumathi et al., 2008). Landfilling sanitary municipal solid waste, just like any other engineering project, requires basic information and planning. The landfill in every area has important effects on environment.

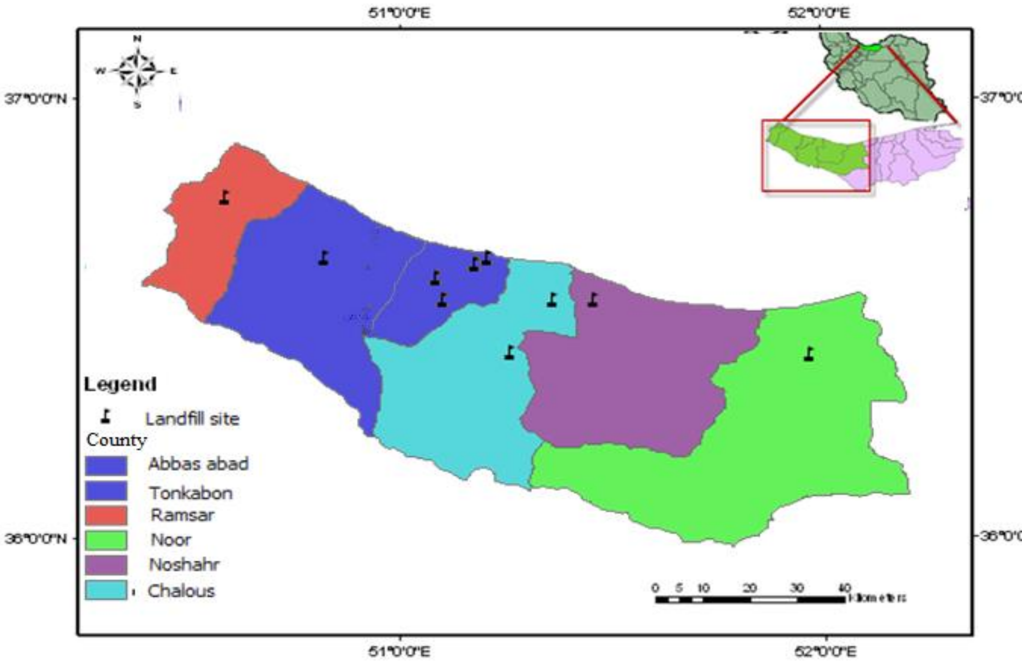

***Figure 1.** Landfill sites in the study area*

Evaluation of the landfill sites using seven standards

The preliminary phase in the environmental impact assessment of landfill site is to know the sensitive parameters. Various methods have been introduced in literatures describing how to select a landfill site (Koshik et al., 2014; Alanbari et al., 2014; Elahi et al., 2014; Salimi et al., 2013; Eskandari et al., 2012; Moeinaddini et al., 2010; Shahabi et al., 2008; Sumathi et al., 2008; Hatzichristos and Giaoutzi, 2006; Heydarzadeh, 2001). The present study applied these methods to evaluate current municipal landfill sites situated in the studied area. In this study, 10 municipal waste landfills (Ramsar, Tonekabon, Abbas Abad, Kelardasht, Salmanshahr, Kelar Abad, Chalous, Marzan Abad, Noshahr, Noor) in 12 municipal districts were initially evaluated using the approved standards based upon the guidelines of British Columbia (BC), Environmental Protection Agency (EPA), Oleckno method, MPCA (Minnesota Pollution Control Agency), Regional Screening, Management and Programming Organization of Iran (MPO) and Iran Department of Environment (DOE). Each method and its criteria are mentioned and compared with each other in *Table 1*, however, the Oleckno index determination is mentioned separately in *Table 2*. In the Oleckno method, the annual average rainfall, soil type and soil depth are the three important

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factors which are considered to determine the rank of each landfill site. For this purpose the following equation was used (Monavari and Arbab, 2005; Salimi et al., 2013):

Oleckno Index Method Score = rainfall (mm/year) + soil type + ground water table (m)

Table 1. *Comparing the seven methods and their criteria*

Criteria methods	Distance to fault	Soil	Groundwater sources of drinking water	Distance to airports	Flood plain, flood basin	Land use
British Colombia			The distance between the discharged MSW and the nearest residence, water supply well, water supply intake, is to be a minimum of 300 meters.	The distance between an airport utilized by commercial aircraft and a landfill containing food wastes which may attract birds is to be a minimum of 8.0 kilometers.	Landfills proposed for locations within the 200 years floodplain and the associated floodway are not to be sited without adequate protection to prevent washouts.	The distance between the discharged MSW and public park is to be a minimum of 300 meters.
DOE			In areas where the water level is high ,it should be a2-meter-deep layer(made of silt and clay)and maximum permeability millionths of a centimeter per second must be provided	Minimum of 8 kilometers		Landfills should not be in conflict with Populated areas or other land uses distance to farmland 500metres, distance to the major cultural, archaeological and historical sites must be suitable
MPO	Minimum distance of 300 meters		Minimum distance of400 meters from the municipal water wells	Minimum of 3 kilometers		
MPCA (Determinative criteria)				Do not cumulate birds in sensitive area around airport	Distance from area with 100 years retention period flood	
EPA	The distance from faults must be at least 60 meters	Distance to areas with unstable soil		Distance within 3048 meters of runway airports turbojet aircraft, the distance of 1524 m from the runway airports piston aircraft	Distance from area with 100 retention period flood	

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Regional screening	The distance from faults must be at least 61 meters	The areas with shortage supply of heavy clay and fine grained soil for using coating layers are not suitable for municipal solid waste landfill sitting.	Regions with high underground water levels are not compatible for MSW sites, if the hydraulic trap method is used. At least 300 meters distance from water wells	At least 3 km distance from the airport.		At least distance of 150 m from, commercial, educational and residential centers and at least 80 m from industrial applications. The agricultural land use can be suitable for solid waste landfill sites

Continue ***Table 1.***

Criteria	Surface water (lake, river, lagoon)	Geology	Distance to residential areas	Distance to road	Preserve and bird habitat
BC	The distance between the discharged MSW and the nearest surface water is to be a minimum of 100 meters.	Landfills are not to be located within 100 meters of an unstable area	The distance between the discharged MSW and the nearest residence, hotel, restaurant, food processing facility, school, church or public park is to be a minimum of 300 meters.		The buffer zone between the discharged MSW and the property boundary should be at least 50 meters of which the 15 meters closest to the property boundary must be reserved for natural or landscaped screening (berms or vegetative screens)
DOE	Landfills should not be located in the wetlands and unique habitats Minimum distance of 2000 meters to surface waters	Not to be Placed on faults, underground mines, subsidence and collapse of cavities	Distance of 10-15 kilometers from the city	Distance of 3-5 kilometers to main road	
MPO	Wetlands should not be selected as the burial place ,landfills must be away from lakes ponds more than300meters.Minimum distance of 100 meters to rivers			Minimum distance of 300 meters	

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MPCA	Minimum 305 meters distance from any lake or pool, Minimum92meters distance from any river or channel, Avoiding from wetlands	Distance from area with limestone caves			
EPA	Landfills should not be located in the wetlands	Distance to high seismic areas, (displacement of rocks and karst areas)			
Regional screening	The MSW landfill sites should not be sited near the surface water (minimum distance of 61 m should be observed).	The regions with slide risk potential and sensitive clays are not suitable for landfill sites. The regions with high sensitive soils such as limestone and fragile soils are not suitable for landfill sites. The MSW landfill site should not be sited in the ravines		A proper distance from the main road should be considered. (Less than one kilometer is ideal)(economic)	

Table 2. *The indices of rank determining in Oleckno method*

The annual average rainfall (mm/year)	**Less than 250 (mm/year)**	**255-760 (mm/year)**	**765-1780 (mm/year)**	
Score	21	7	6	
Soil type	Clay silt or clay and sand	Silt and soft sand	Mud	Gravel and cobble
	12	5	4	0
Soil depth(m)	1.5 - 3	3 - 6	6 - 9	9 <
Score	3	7	9	9
Oleckno rank	Good	Acceptable	Unacceptable	
Score	24 - 42	21-23	>20	

The present study consisted of two phases; first phase included determining the characteristics of the 10 landfill sites by reviewing the library data, previously published literatures and using the ArcGIS software maps (version 10.2). The second phase is to address the locally preferred criteria for landfill site evaluation in the northern Iran for Caspian Landfill Criteria (CLC) model.

Most of the information was obtained from the Mazandaran Management and Planning Office of the Governor with a scale of 1:100000. The hydrology and hydrogeology maps (surface and groundwater maps) with a scale of 1:250000 were

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obtained from the Geographic Information Centre of the Mazandaran Regional Water Organization. By locating the GPS coordinates of the available landfill sites in field view and entering them as latitude and longitude in the GIS software database the landfill site map layer was prepared. The gathered data were then converted into a point data. Thematic maps, characterizing the affecting factors, were generated for landfill sites evaluation (Yazdani et al., 2013). The map layers of the evaluation criteria in the study area are shown in *Fig. 2*. The steps of the first phase of the present research are illustrated in *Fig. 3*. All the mentioned steps are considered in each of the seven evaluation methods for total research area.

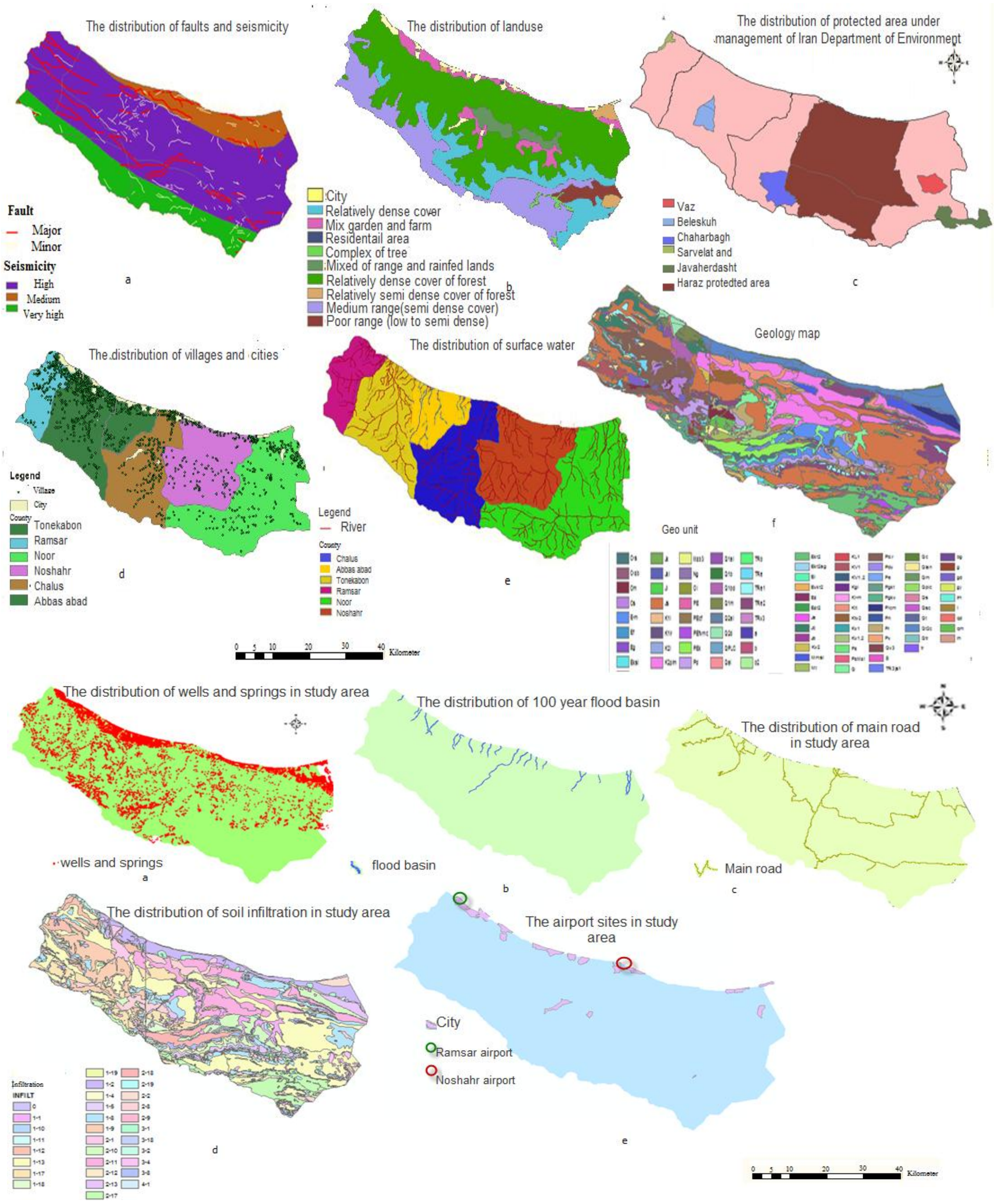

***Figure 2.** Criteria maps in the study area*

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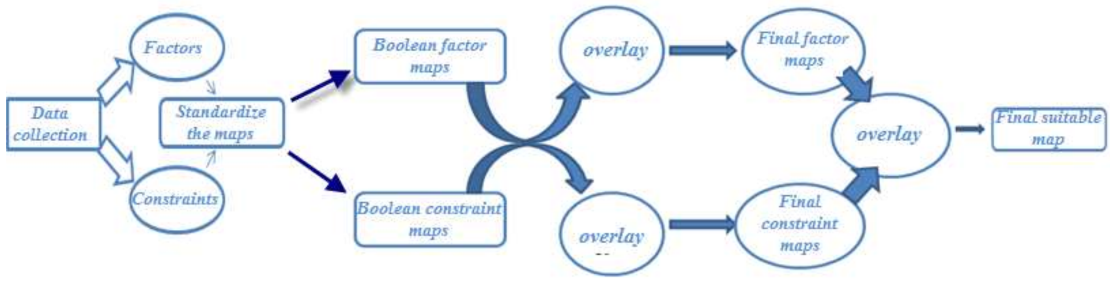

Figure 3. *The research steps in first phase (Yazdani et al., 2015a)*

There were two types of map in first phase of the study; factors (such as faults map and landuse), constraints (such as distance to spring, distance to road, distance to rivers and distance to protected areas). Since there are some regulations applying these seven methods to evaluate landfill sites, Boolean logic was used to standardize the constraint and factor of the map layers. Therefore, all the areas that are impermissible for landfill site sitting according to the 7 guidelines and their principals (constraints) as well as whole areas that fall inside the restricted area (buffers) which landfill site development is prohibited in ArcGIS software with the reclassified module were determined. In map layers the value of the restricted area (unsuitable area) was 0 and that of the other area was 1 (suitable area) (Yazdani et al., 2015a). Different criteria map layers were prepared according to the extant standards which are mentioned in *Table 1*. Buffer maps using the buffer option in ArcGIS were prepared for various criteria. The areas falling inside the buffer areas are unsuitable for municipal solid waste landfill site sitting.

The GIS-based constraint mapping technique was then used to evaluate the suitability of each existing landfill site in studied area considering all the mentioned criteria in each method. The results are shown in *Table 3*.

The overlaying of Boolean factor maps is shown in *Fig. 4* and *Fig. 5* shows the overlaying of Boolean constraint maps in Iran Department of Environment (DOE) method. The final suitable map according to DOE method is shown in *Fig. 6*.

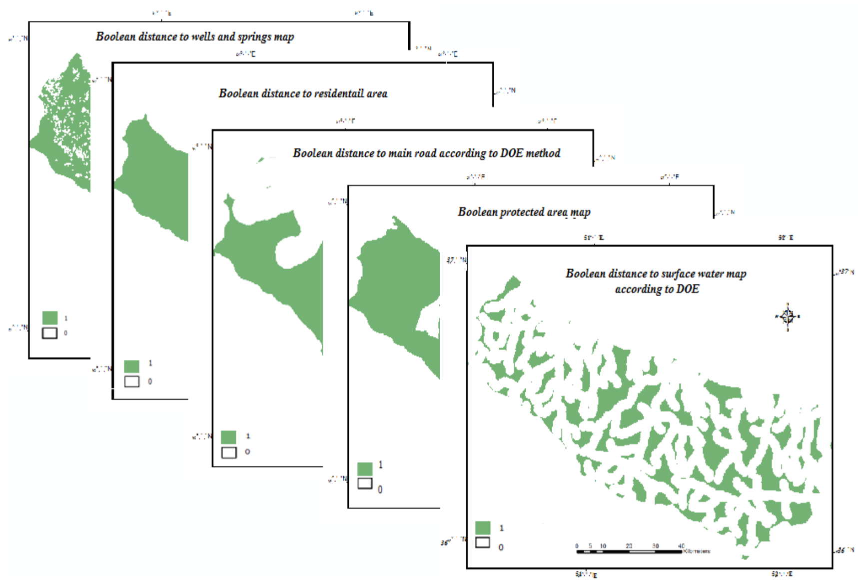

Figure 4. *Overlay of Boolean constraint maps to achieve the final suitable map in DOE method*

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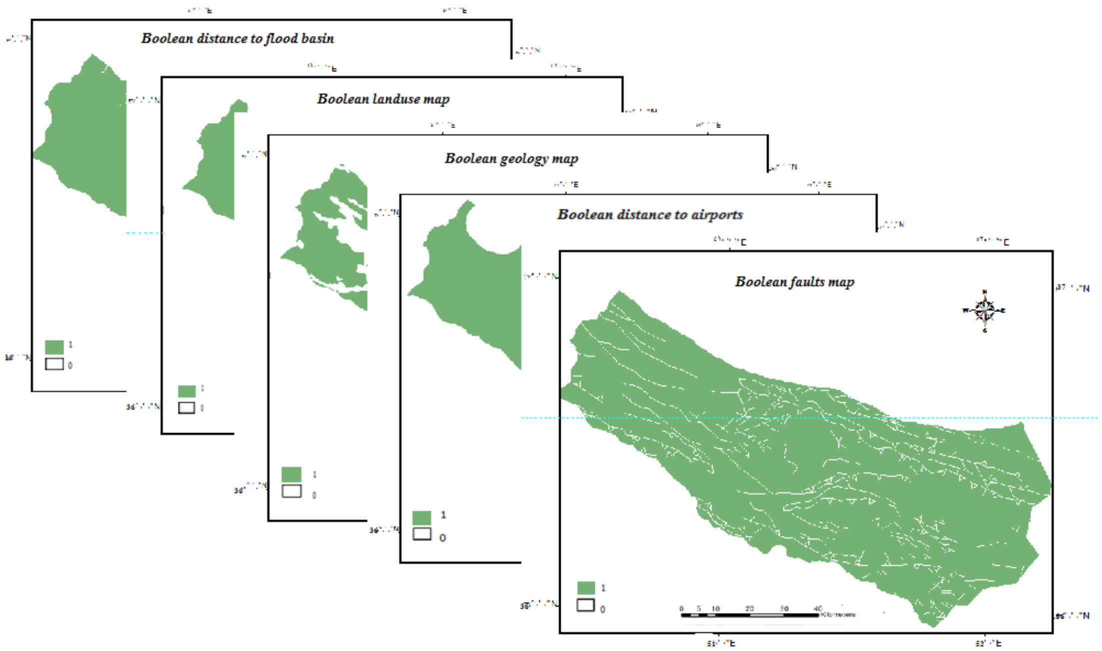

Figure 5. *Overlay of Boolean factor maps to achieve the final suitable map in DOE method*

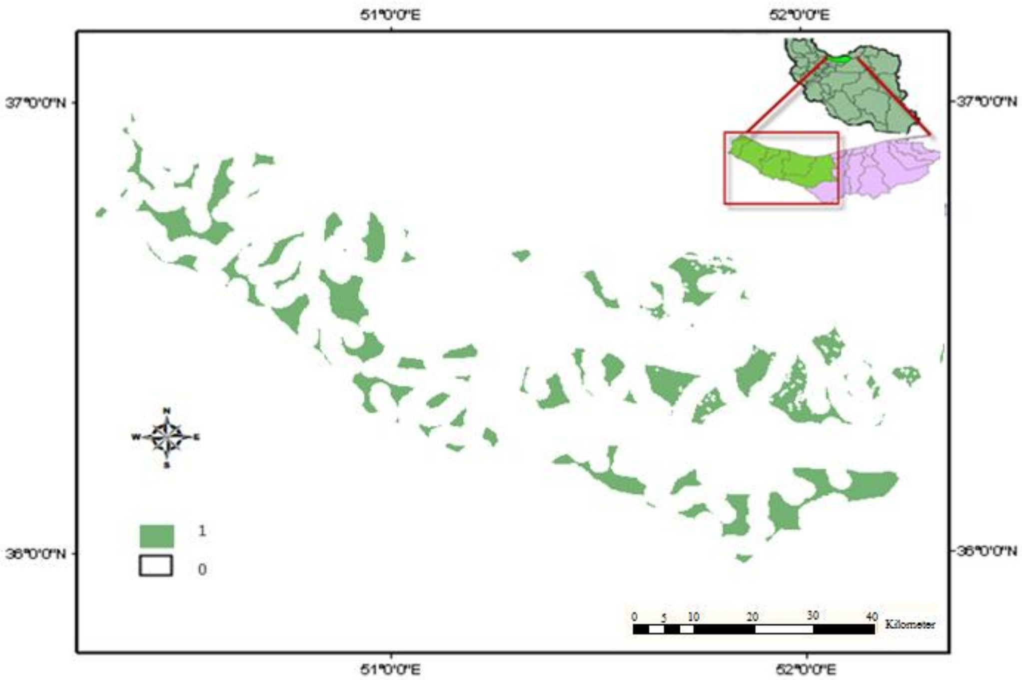

Figure 6. *Final suitable map according to DOE method after overlaying Boolean constraint maps and Boolean factor maps*

Evaluation criteria for Caspian Landfill Criteria (CLC) model

The second phase of the study consisted of four main stages to select and evaluate landfill site based on the Caspian Landfill Criteria (CLC) model; choosing evaluation criteria using Delphi method, standardizing map layers with Boolean logic and fuzzy

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functions, application of Analytical Hierarchy Process (AHP) method to identify the importance of the selected criteria and finally combining the information gathered from various criteria to combine a single evaluation index with Multi Criteria Evaluation (MCE).

Table 3. *The suitability of landfill sites with 7 methods in first phase*

Landfill site name	Regional screening suitability	BC suitability	EPA suitability	MPO suitability	DOE suitability	MPCA suitability	Oleckno suitability
Ramsar	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Suitable	Suitable
Tonekabon	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Suitable	Suitable
Abbas abad	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Suitable	Suitable
Kelardasht	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Suitable	Suitable
Salmanshahr	Unsuitable	Unsuitable	Unsuitable	Unsuitable	Unsuitable	Unsuitable	Suitable
Kelar abad	Unsuitable	Unsuitable	Suitable	Unsuitable	Unsuitable	Suitable	Unsuitable
Chalous	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Suitable	Suitable
Marzan abad	Unsuitable	Suitable	Unsuitable	Suitable	Unsuitable	Suitable	Suitable
Noshahr	Unsuitable	Unsuitable	Suitable	Unsuitable	Unsuitable	Unsuitable	Unsuitable
Noor	Suitable	Suitable	Suitable	Unsuitable	Unsuitable	Suitable	Unsuitable
Suitability percent of the total study area	58.17	62.38	77.9	48.5	20	95.32	38.9

Choosing the evaluation criteria

At first, the Delphi technique was used to identify the suitable criteria. This method is an effective tool to achieve a well-thought-through consensus among experts. Delphi technique has been applied in MSW landfill site sitting in the past (Sumathi et al., 2008; Hatzichristos et al., 2006; Koshik et al., 2014). Therefore, the findings of twenty experts in the field, with the most relevant experience, have been included to determine which factors should be considered for alternative ranking. This was accomplished using questionnaire form. A list of criteria based on the 7 mentioned guidelines, as well as a review of the scientific periodicals on previously works was conducted in the questionnaire form. The experts who were familiar with the studied area and participated in the study were requested to supply a list of the preferred evaluation criteria for landfill site selection and evaluation. To data, 18 important criteria have been determined to evaluate landfill sites, and thus, were prepared as input map layers.

Standardizing map layers

Considering the fact that to measure the criteria a variety of scales are used, therefore, it is necessary that the values present in layers of different criteria could be changed into proportional and comparable units. The map layers were standardized in a GIS environment using fuzzy and Boolean logic functions. Boolean logic was used to standardize the constraint map layers. Therefore, in ArcGIS software with the reclassified module was determined. In map layers the value of the restricted area (unsuitable area) was 0 and that of the other area was 1 (suitable area) (Yazdani et al., 2015a). To standardize the factor map layers the criteria-related fuzzy approach was used. To make fuzzy factor maps the threshold should be determined for the values of the criteria, the type and shape of the membership function which are shown in *Table 4* are required.

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The maps were standardized in the 0-255 range for each criterion with 0 as the least and 255 as the maximum suitability range. The linear function which is provided using IDRISI software is applied in the present study.

Due to its high capabilities, IDRISI software was applied to calculate the weights of the criteria, to standardize the criteria by fuzzy functions and also to merge the criteria by MCE model. Therefore, IDRISI software is a suitable option for decision-making using spatial information (Moeinaddini et al., 2010). To quantify the fuzzy diagrams (membership functions), linear scale conversion method was used based on minimum and maximum values as scaling points. In monotonically increasing functions, the linear scale transformation method was used as shown in equation (1) (Eastman, 2012).

$$X_i = 1 - \frac{(Ri - Rmin)}{(R_{max} - R_{min})} * \text{standardized\_range} \quad \text{(Eq.1)}$$

$$X_i = \frac{(Ri - Rmin)}{(R_{max} - R_{min})} * \text{standardized\_range} \quad \text{(Eq.2)}$$

where:
X_i is pixel value after standardization,
R_i is pixel value before standardization,
R min is minimum point in factor,
R max is maximum point in factor,
Standardized _range is range standardization (on a scale of 255 bytes).

In monotonically decreasing linear functions, linear scale transformation method was sued based on equation (2). In symmetric (trapezoidal) functions, a combination of equations (1) and (2) was used. All these steps were carried out in the ArcGIS and IDRISI software with Con conditional statement. In the case of discontinuous functions, such as land use and geological factors, the fuzzy values associated with each class were determined using equation (1).

Table 4. *Factors used to form the landfill site sitting suitability map, with indications on their endpoints (the 2nd one shows to the highest suitability value and the 1st endpoint to the lowest) and their comparative weight, M.I (Monotonically increasing), M.D (Monotonically decreasing) and S (symmetric)*

Criteria [unit of measurement]	End point 1	End point 2	Weight	Fuzzy function
Distance from population center [m]	5000 9000	7500	0.109	S
Soil depth [qualitative classes]*	1	5	0.034	M.I
Distance from sea[m]	3000	5000	0.082	M.I
Distance from faults [m]	1000	3000	0.016	M.I
Bedrock material [qualitative classes]*	1	5	0.015	M.I
Soil infiltration	1	5	0.054	M.I
Distance from industrial center[m]	300	600	0.041	M.I

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Distance from surface water [m]	3000	4500	0.112	M.I
Distance from airport [m]	3000		0.040	M.I
Distance from main road [m]	3000 10000	5000	0.040	S
Distance from wetland, lake [m]	500	1000	0.059	M.I
Distance from sensitive ecosystem [m]	500	1000	0.029	M.I
Slope [percent]	40	20	0.015	M.D
Land use [qualitative classes]*	1	5	0.029	M.I
Distance from flood basin [m]	2000	5000	0.061	M.I
Soil texture [qualitative]*	1	5	0.049	M.I
Ground water table [m]	5	10	0.049	M.I
Distance from underground water sources [m]	500	1000	0.109	M.I

* are mentioned in *Table 5*.

Application of AHP method

After the GIS database for landfill site sitting and the thematic map layers for each criterion were prepared and their importance were identified, a relative classification was carried out for each factor based on the relative influence of each criterion. In this study, analytical hierarchy process (AHP) was applied for pair-wise comparison to create comparative weights. In the AHP, the first step is the decomposition of a difficult complex decision into the easier decision subject to form a hierarchical model. In each hierarchical model, the upside level is the final goal (in this study the goal is landfill site evaluation). In analysis step, simultaneous pair wise comparisons between each both criteria and their relative values was carried out using the Expert Choice software for a simple classification. The comparison matrix was developed for eighteen criteria. The criteria weights are shown in *Table 4*.

Combining criteria using MCE (Multi Criteria Evaluation)

Multi criteria evaluation is mainly used to incorporate the different criteria to form a single evaluation index (Voogd, 1983). After standardization of criteria maps (factors and constraints) and determining the weights factors, the next step was to perform multi-criteria evaluation process. The Boolean intersection logic, AND logic or the multiplied or logic function according to equation (3) were used to integrate layers of constraints and provide the final layer. All criteria weights were considered equal (Eastmen, 2012).

$$C = \Pi c_j \qquad \text{(Eq.3)}$$

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where C is final constraints, Π is multiplied index, cj is constraints criterion j score.

Considering the fact that, the Weighted Linear Combination (WLC) is one of the best and useful methods of multi-criteria decision making (Heydarzadeh 2001), was used throughout this study. In order to perform the assessment process using this method in the present study, each factor (criterion) was multiplied by its corresponding weight according to the equation (4).

The unsuitable areas were omitted by summing the gathered results and multiplying the constraints. Consequently, the suitable area(s) for landfill sitting was obtained.

$$S = \Sigma \, WiXi\Pi cj \qquad \text{(Eq.4)}$$

where S is suitability, Wi is weight of factor i, Xi is fuzzy value of factor i, Π is multiplied index, Cjis is constraints criterion j score.

WLC approach was applied in IDRISI software according to equation (2). After generating a raster map in ArcGIS software (this map is based on the pixel level) using the "Re class" command, (Re class), the final plotted map was divided to 5 sections from 0 as the minimum to 250 as the maximum suitability rate.

Results

After reviewing each waste deposit site, considering all the criteria in every 7 methods applied in the present study, the incoherence in suitability was apparent in first phase. For example, some sites were suitable using one method, but unsuitable using another. The suitability report of the studied landfill sites is shown in *Table 3*. Thus, presenting unique locally parameters suited to particular ecological conditions, seems necessary for evaluating all landfill sites. To achieve a comprehensive and applicable evaluation, the criteria should be defined in accordance with the locally condition of the studies area. The review of all existing methods has shown that none of them have considered the distance from sea, which is critically important for the entire region. Considering the importance of high priority data layers in MCE, the weighting of these layers and the fact that each of these criteria and their importance changes in accordance with the special environmental conditions, it is necessary to localize the criteria for different environmental zones. Therefore, 18 factors were used for alternative ranking (shown in *Table 4*) in CLC model. The map layers of each of these 18 factors are essential to be thought out. Consequently, the eighteen essential evaluation criteria including their regulations and constraints were prepared based on the prior inspections, present regulations, point of view and questionnaires answered by twenty specialists familiar with the field of study and also familiar to studied area (the Delphi method).

Based on the availability of the data, these 18 important criteria were modified in order to evaluate the landfill sites, as input map layers. In the present study, a GIS/MCE integrated method has been used for the data analysis. Furthermore, the results were compared and the accuracy was checked using an AHP/Fuzzy integrated method. *Table 4* indicates that the fuzzy approach was applied for standardization and the weights of each criterion (preferred value). The description of qualitative criteria is indicated in *Table 5*. Five standard maps out of eighteen are showed as samples in *Fig. 7*. The standardized maps were divided into 5 classes in the 0-255 range for each criterion with 0 (unsuitable) to 255 (most suitable areas). In this study, a multi-criteria evaluation approach integrated with Arc GIS overlay analysis was used to choose the most

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preferable landfill site in studied area. The suitable regions were recognized for landfilling considering the final map layer. In executing the CLC model, the important of the weights and the preferred value of each of the criterion are critical.

In the present study, parameters and their prioritization includes distance to river, distance to population centres, distance to springs and wells, distance to sea, flood plains, distance to lakes and lagoons, the rate of soil infiltration, soil texture and underground level, distance from the airports and main roads, the depth of soil, distance from sensitive habitats and land use, distance from fault and, the least important one, the stone material of context and slope.

The results of this study have been achieved using fuzzy multi-criteria decision making and analytic hierarchy process, which are similar to that used in researches on locating and evaluating landfill sites. This integrated method can be used in areas similar to those in this study or, to be more general, in the coastal regions of the Caspian Sea.

Table 5. *Qualitative criteria description*

Criteria	1	2	3	4	5
Suitability range	Very unsuitable	Unsuitable	Low suitability	Suitable	Very suitable
Bed rock material	Permeable flood basin floor	Sand, stone, limestone, dolomite, deposits range, conglomerates, alluvial fans, alluvial present covenant	Igneous and metamorphic rocks with low breakage and silt	Schist, clay ,tuff, evaporated rock, clay and mud, fine loss	Shale, marl and clay
Soil type (depth and texture)	Shallow to moderately deep soils with limestone, or with gravel bearing	Shallow to moderately deep soils, medium to heavy pebble texture on the rocks, moderately deep to deep loamy to gravelly soil	Deep heavy texture soils in some areas with limestone floor concentration	Deep soil with moderate to heavy texture	Deep soil with heavy texture, moderately deep to deep soils
Land use	Forest, dense range, populated area, river floor	Very low densities of forest ,agricultural lands, Gardens	Semi dense range, agricultural land, incorporating gardens	Poor range, low density other land	Land without vegetation, rocky protrusions
Infiltration	Very high	High	Medium	Low	Very low

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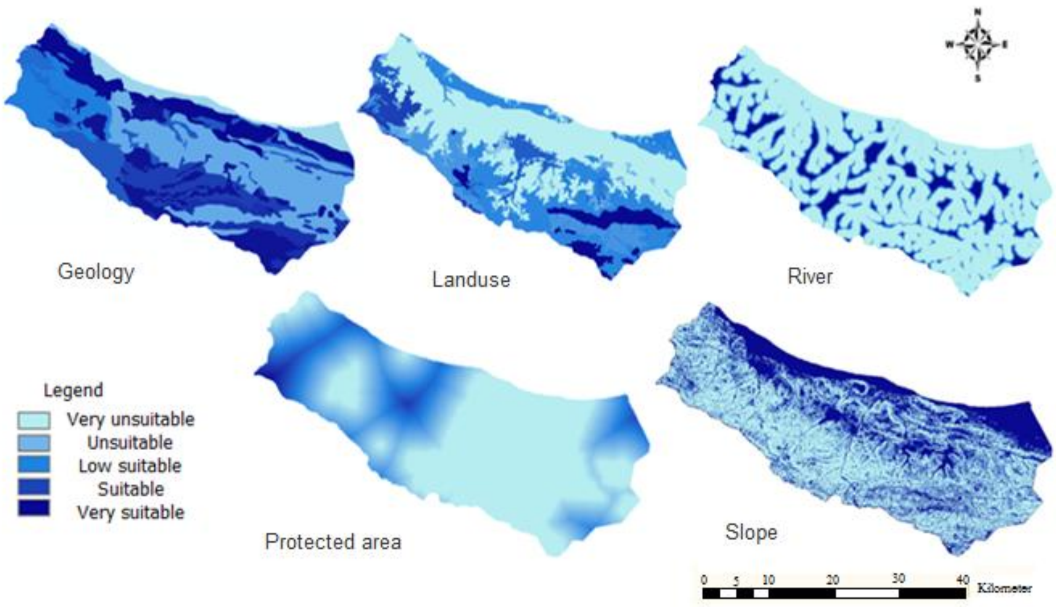

Figure 7. *Some of the standardized maps in phase 2 according to fuzzy logic*

Discussion

Unfortunately in Iran, similar to other developing countries, there have been few studies carried out on evaluating current landfill sites. Municipalities rarely consider the ecological properties of an area, adequately, in accordance to the governmental organizations and international standards and the pre-defined criteria before depositing the waste material at the edges of the cities and degrading the valuable natural ecosystems. Therefore, the applicable criteria are hardly used in most of the MSW landfill sites. Comparing the applied criteria indicated that all the criteria have some similar aspects in common. According to the previously studies, some parameters are joined in all methods. These common parameters have been considered in the present study. Parameters such as hydrology, distance form roads and airports and preserving special habitats are some of the factors taken into account in most of the methods due to their crucial significance. The studied parameters and their prioritization can be different based on the ecological characteristics. Choosing a set of effective parameters for selecting the location and evaluation of the landfill sites in relation to the environmental conditions of the studied area is critical and has a direct effect on prioritizing parameters (Sener, 2004). For instance, one study has shown that the highest prioritised parameters in landfill site selection are distance from urban and rural areas, surface water, geology, land use and fundamental instalments and roads, respectively (Khan and Anjaneyulu, 2003). In another study the priority of parameters was distance from geologic faults and the depth of earth, distance to the airports, distance to urban areas, distance to lakes, dams and slope of the soil, land use, paths network and in final run the least priority belonged to instalments and telecommunication (Delgado et al., 2008). In this study, preservation of the ecosystems of the vast water resources, both underground and surface, as well as the Caspian Sea was the preeminent concern. (Tajziehchi and Monavari, 2013) mentioned in their research that geographical features and special environment in this region are the main reasons for the complex landfill site sitting process. All of the considered criteria in this study and previously researches are

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to prevent land degradation and protect the environment. To evaluate the landfill sites in the studied area in the present research, which is situated in western part of Mazandaran province, noble approaches are required because of its special topographic conditions (closeness to the forest and the sea), high level of underground water and high rate of tourism (especially in spring and summer). Considering the importance of prioritizing the data layers in multi-criteria evaluation and defining the value of the layers based on their specific ecological conditions, the criteria and their importance can be changed. Therefore, locally criteria—Caspian landfill criteria—appropriate to the ecological condition of the area has been presented.

Conclusion

This research shows the incoherence in suitability status of the 10 current landfill sites in west area of Mazandaran province with using seven different landfill site sitting approaches. In second phase, the proposed Caspian Landfill Criteria with effective and native criteria has been used to determine the status and (un)suitabilty of the current landfills and the studied area. The suitability of the CLC (Caspian Landfill Criteria) model for landfill site sitting and evaluation of the current landfill sites were considered through field observations. Finally, only about 0.4% of the whole studied area was appropriate for landfilling. The most principal parts in the CLC model were the importance of the weights of the criteria and the preferred weights of them which in this research, distance to residential area parameter has the highest and slope has the least weight. As the findings of the present study have revealed, there are insufficient suitable areas in the western part of Mazandaran province for landfill site sitting. Furthermore, landfilling is not an appropriate approach for waste disposal in this region. There for other approaches should be considered for waste disposal with regard to the environmental characteristic of this specific region. The results of this study have been achieved using the same method applied for processing the consequent and phasic analysis in multi-criteria decision making in researches on locating and evaluating the landfill sites. The Caspian Landfill Criteria model with effective and native criteria has been used to determine the status of the current landfills and recognize the ruined areas, which are a good illustration of land degradation, in order to achieve an optimal management approach to convert the land into a sustainable natural ecosystem of the southern coast of the Caspian Sea.

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RESPONSE OF SWISS CHARD AND SOIL PROPERTIES TO CO-APPLICATION OF DIFFERENT FERTILISERS WITH EFFECTIVE MICROORGANISMS

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Abstract. The use of mineral fertilisers causes soil degradation; however, there are alternative systems such as the application of effective microorganisms (EM), although this technology is not fully studied in Africa. The objective of this greenhouse experiment was to assess the response of Swiss chard (*Beta vulgaris* subsp. cicla) and soil chemical properties to EM. The crop was harvested twice - after eight and sixteen weeks. The experimental design was randomised complete block design, treatments were: control, EM, recommended fertilizer (RF), RF + EM, compost, compost + EM, 1/2 RF + compost, 1/2 RF + compost + EM, goat manure (GM), GM + EM, 1/2 RF + GM, and 1/2 RF + GM + EM, with six replications and each pot represented an experimental unit. The first yield was higher than the yield from the second harvest, except for the GM treatments due to nitrogen immobilisation. Yield was improved by 32.5% for the second harvest where GM was applied owing to improved nutrient availability. The application of fertiliser doubled the yields, which were not reduced when half of the recommended fertiliser was applied with GM or compost. The results confirm that EM's application had inconsistent effects on Swiss chard yields and soil properties.
Keywords: *Beta vulgaris subsp. cicla, mineral fertiliser, organic manure, effective microorganisms, soil properties*

Introduction

The use of effective microorganisms (EM) in agricultural soils as an amendment for disease control and maintenance of healthy resilient soils was reported by Daly and Stewart (1999), and Hu et al. (2013). EM are a microbial culture of a naturally occurring assortment of beneficial microorganisms such as photosynthetic bacteria (*Rhodopseudomonas plastris* and *Rhodobacter sphacrodes*), lactic acid bacteria (*Lactobacillus plantarum*, L. casei, and *Streptococcus lactis*), yeast (*Saccharomyces* spp), actinomycetes (*Strptomyces* spp.) and fermenting fungi that coexist together (Bell et al., 2013; Hu and Qi, 2013; Boga et al., 2014; Daur et al., 2015). EM are used as an inoculant to increase the microbial biomass diversity of soils through a rapid proliferation of their constituents (Javaid and Bajwa, 2011; Bhalla et al., 2013; Khan et al., 2013). Application of EM solution to soils enhances the growth of other microorganisms and a colony forming units (CFU) of 110000000 per gram on TS agar has been recorded (Javaid and Bajwa, 2011).

EM improve crop quality, growth and yield through effective mineralisation of soil organic matter (Megali et al., 2014; Daur, 2015, 2016; Xu et al., 2016) and are applied

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with a carbon and energy source of molasses for the microorganisms (Mayer et al., 2010; Ahn et al., 2014). The integrated use of EM with organic amendments is an effective technique for enhancing nutrient release and supply from the sources. The mechanism of EM activities for rapid nutrient release from organic amendments involves a rapid proliferation of its effective and beneficial microorganism content within the soil system (Bouws and Finckh, 2008; Gabhane et al., 2012).

Some studies have shown that the inoculation of soils with EM can improve soil and crop quality (Javaid and Bajwa, 2011; Megali et al., 2014; Javaid and Bajwa, 2016). Research and field testing of EM were conducted in the Asia Pacific region (Bajwa et al., 1995; Bajwa et al., 1999; Hussain et al., 1999; Iwaishi, 2000; Hu and Qi, 2013; Daur, 2015, 2016; Xu et al., 2016), Switzerland (Mayer et al., 2010; Megal et al., 2013), Africa (Ncube et al., 2011; Ncube and Brutsch, 2012) and New Zealand (Daly and Stewart, 1999) where the application of EM to onions, peas and sweet corn increased yields by 29%, 31% and 23%, respectively. Khaliq et al. (2006) reported that the integrated use of compost with EM increased seed cotton by 44% over the control treatment. The application of EM with 50 Mg ha^{-1} of animal manure and 30 mg ha^{-1} of a combination of various green crop residues and weeds separately, increased the production of polysaccharides, alkaline phosphatase and esterase enzymes (Valarini et al., 2002). The application of compost A with EM resulted in the highest strawberry yield and quality (Hammad et al., 2014). In a study conducted by Megali et al. (2014), application of EM increased plant growth fruit production by 61% and plants sustained 25% higher insect survival.

EM are effective in improving plant and soil quality, but studies conducted at the University of Fort Hare (Ncube et al., 2011; Ncube and Brutsch, 2012) did not detect a substantial contribution to crop yield by the recommended application of EM in combination with commercial compost. This was attributed to the low-quality C constituents in the compost used, which is typical of most compost that have matured. Composted organic wastes are low in soluble C, because microorganisms use most of it during the composting process (Groenestein and van Faassen, 1996; Boechat et al., 2013). Therefore, mature compost such as the one used in the earlier study may not be able to effectively support proliferation of the decomposer community, including EM.

The objective of the study was to evaluate the single and integrated application of EM with fresh and composted organic sources of nutrients on the yield of Swiss chard (*Beta vulgaris* subsp. cicla) grown in Oakleaf soil in pots.

Materials and methods

Soil, goat manure and the compost characteristics

The soil used in this study was from the research farm of the University of Fort Hare in Alice, Eastern Cape, South Africa. The farm is located at 32° 47′ S and 26° 50′ E and 535 m above sea level (asl.). The farm is situated in a semi-arid ecological zone that has an average annual rainfall of approximately 575 mm during the summer and a mean daily temperature of 22.5°C during the day and 18.8°C at night. In winter the temperatures are about 13.6°C during the day and below 10.3°C at night (Marais and Brutsch, 1994). The soils are of an Oakleaf form (Oa), belonging to the Jozini series (Soil Working Group, 1991). The soil characteristics are as shown in Table 1. An equivalent of 30 t ha^{-1} of goat manure was applied (which supplied 657, 30, 135 kg ha^{-1} of N, phosphate (P), and potassium (K), respectively) (*Table 1*). Thirty t ha^{-1} of nature's

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grow compost were applied (which supplied 60, 15 and 12 kg ha^{-1} of N, P, and K, respectively) (*Table 1*).

***Table 1.** Selected properties of the experimental soil (upper 0–30 cm depth), goat manure and compost*

Characteristics	Soil	Goat manure	Compost
pH (1:2.5 soil: water)	6.1	8.0	4.3
EC (µScm^{-1})	90.0	2.2	2.4
CEC_{sum} (meq/100g)†	12.1	-	-
Exchangeable acidity (cmol/L)	0.1	-	-
Total cations (cmol/L)	12.1	-	-
Total N (g kg^{-1})	0.6	21.9	3.2
Total P (g kg^{-1})	-	1.0	0.5
Available P (g kg^{-1})	0.4	-	-
Total K (g kg^{-1})	5.0	4.5	0.4
Exchangeable K (g kg^{-1})	0.1	-	-
Exchangeable Ca (g kg^{-1})	1.7	-	-
Exchangeable Mg (g kg^{-1})	0.3	-	-
Total C (g kg^{-1})	9.4	426.3	96.65
Organic C (g kg^{-1})	6.0	-	-
C : N	16.5	19.5	30.2
C: P	-	426.3	386.6

†CEC is cation exchange capacity, and EC is electrical conductivity

Treatments and experimental design

The experiment was conducted in a glasshouse whose conditions are controlled at the University of Fort Hare, South Africa, and was laid as a randomised complete block (RCB) with six replicates. Treatments were: control (T1), EM alone (EM) (T2), recommended fertiliser (RF) (N 150: P 90 kg ha^{-1}) (T3), RF + EM (T4), compost (Comp) (T5), comp + EM (T6), 1/2 RF (N 75: P 45 kg ha^{-1}) + comp (T7), 1/2 RF + comp + EM (T8), goat manure (GM) (T9), GM + EM (T10), 1/2 RF + GM (T11) and 1/2 RF + GM + EM (T12). Two plants of Swiss chard were planted in each pot with a depth of 30 cm containing 15 kg of soil as an individual experimental unit. The soil moisture was kept at field capacity during the course of the experiment. Swiss chard was used in this study due to an increasing awareness on the importance of leafy vegetables in the daily diet which has prompted an increasing demand for the fresh vegetable crops such as Swiss chard throughout the year in South Africa (Abdel-Rahman et al., 2014). Moreover, Swiss chard is known for its high nutritional value due to its high levels of antioxidants and vitamins (Mitic et al., 2013). The crop was harvested at the eighth and the sixteenth week. Inorganic fertiliser was incorporated into the soil, with N applied in the form of NPK prior to planting and as LAN three weeks after transplanting.

Effective microorganisms (EM)

Multiplied - EM, EM - F.P.E, EM 3-in-1 and EM -5 were the EM products used. The Multiplied-EM was dissolved in water in a ratio of 1:300 and applied as a soil drench at a rate of 200 L per experimental unit, 7 days prior to transplanting of Swiss chard.

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During the experiment, multiplied - EM solution, in a ratio of 1: 500, was applied to respective EM - treated units at a rate of 50 L per week. Mixtures of EM - FPE, EM 3-in-1 and EM - 5, mixed at a ratio of 1: 1: 1, then diluted with water at a ratio of 1: 800 were sprayed to control diseases and pests in EM treated units (Ncube et al., 2011).

Soil and leaf analysis

Soil and leaf samples were taken at harvest to assess the treatment effects on soil and plant nutrient content. For leaf sampling, the youngest mature leaves were taken from the top of plants (Hue et al., 2012). The leaf dry matters were dried in an oven at 65 °C and were ground in a hammer mill to pass through a 1 mm mesh sieve. Total P and K contents were determined as described by Okalebo et al. (2002). Total nitrogen was determined using a LECO TruSpec C/N auto analyser (LECO Corporation, 2003).

Crude protein is an estimate for total protein. Crude Protein (CP) is based on a laboratory nitrogen analysis, from which the total protein content can be calculated by multiplying the nitrogen figure by 100/16 or 6.25. This is from the assumption that nitrogen is derived from protein containing 16 % nitrogen (Okalebo et al., 2002). Therefore, crude protein was calculated by multiplying total N x 6.25.

Soil samples were air-dried for two weeks and then were ground to pass through a 2 mm mesh sieve. Soil pH and EC were determined in water extracts as described by Okalebo et al. (2002). Total N and C were determined using a LECO TruSpec C/N auto analyser (LECO Corporation, 2003) and extractable P and K were determined following the Ambic - 2 extraction method (Non-Affiliated Soil Analysis Work Committee, 1990).

Data analysis

The data were subjected to analysis of variance (ANOVA) using the SAS statistical package and means were compared with Tukey test ($p \leq 0.05$).

Results and discussion

Effects of amendments on dry matter yield of Swiss chard

Yield obtained during the second harvest was lower than yield obtained during the first harvest. Yield decreased by 46.2%, 44.2%, 51.3%, 56.6%, 44.1%, 44.3%, 68.0%, 67%, 43.3% and 57.9% for T1, T2, T3, T4, T5, T6, T7, T8, T11 and T12, respectively (*Figure 1*). The application of goat manure with or without EM significantly increased yield for the second harvest. An increase in yield of 61.3% and 84.3% for the second harvest was observed with T9 and T10 relative to the control (*Figure 1*). The positive effects of incorporating goat manure with or without EM observed during the second cropping suggests that soil production was better maintained under goat manure treatment, possibly as a result of nutrients being slowly released over a period of time as suggested by Cooke (1972; Neina et al., 2016a). Application of goat manure to soil decreases nutrients sorption capacity and increases their availability to plants. This is thought to result from the cumulative effects of various processes such as preliminary soil incubation, soil microbial activities, moisture content, incubation temperature, soil pH, N mineralisation and ammendment biochemistry. The immobilisation of nutrients by native soil microbial biomass in response to added manure may also increase its availability to plants. Native microbial biomass apparently increases nutrients

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availability by immobilizing soil inorganic nutrients and releasing them at a later stage through mineralization during microbial biomass turnover. The nutrients is released slowly and taken up by the crop more efficiently and may account, at least in part, for the observed synergistic effects on crop growth observed (Gichangi et al., 2010; Calvalho et al., 2013; Neina et al., 2016b). Therefore, nutrients contained in manure are primarily organic and must be mineralised before they are used by plants.

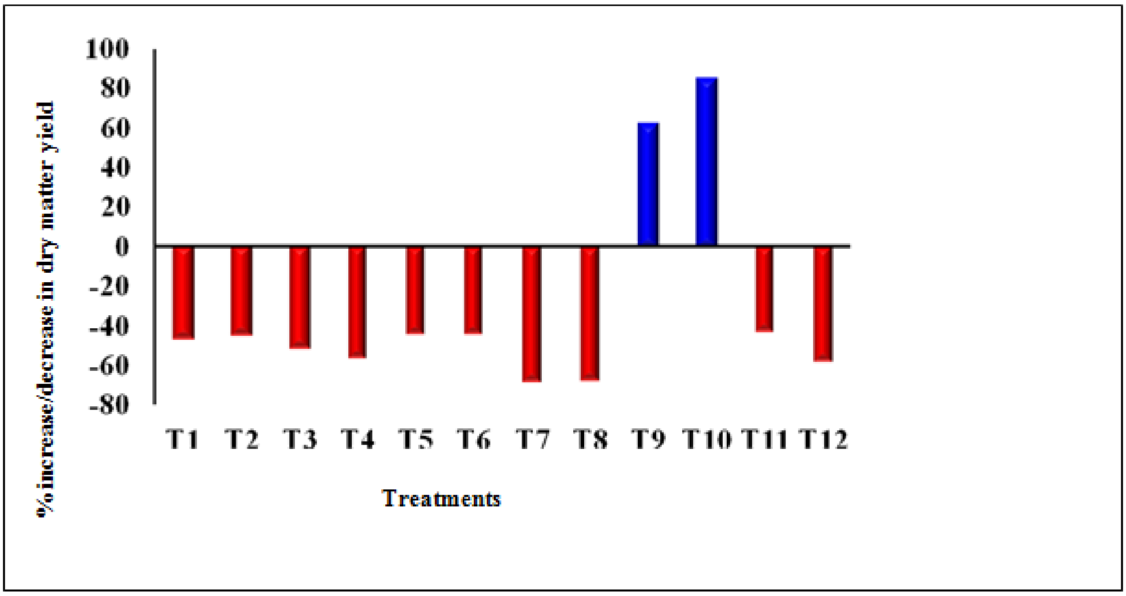

Figure 1. *Effects of amendments on dry matter yield of Swiss chard*
T1*: control,* ***T2****: effective microorganisms,* ***T3****: reference fertiliser,* ***T4****: reference fertiliser + EM,* ***T5****: compost alone,* ***T6****: compost + effective microorganisms,* ***T7****: half reference fertiliser + compost,* ***T8****: half reference fertiliser + compost + effective microorganisms,* ***T9****: goat manure alone,* ***T10****: goat manure + effective microorganisms,* ***T11****: half reference fertiliser + goat manure,* ***T12****: goat manure + half reference fertiliser + effective microorganisms*

The performance of EM and compost was not as expected when applied alone or in combination (*Figure 2*) and their performance was speculated to have been affected by the soil organic C, which was low (0.6%). In soils with low organic C, release of nutrients from compost materials is relatively low and cannot increase yield to the same level as the mineral fertilisers. The added compost in soils with low organic C could be first utilised by native soil microbes, as plants and microbes compete for applied N (Khaliq et al., 2006). Unlike microbes, plants require more energy for nutrient absorption and uptake so that microorganisms become successful in utilising most of the applied N. The release of nutrients from compost materials, their absorption by plants and the remineralisation of immobilised nutrients require time and might not be attained within a few weeks (Hussain et al., 1999).

With regards to the performance of EM, researchers have shown that it is difficult to establish the predominance of EM cultures in soil within a few weeks. Indigenous soil microbes often affect the establishment of EM negatively (Bajwa et al., 1995). However, the problem can be overcome through prolonged, repeated applications of EM (Javaid et al., 2000). Soil type, the source and amount of soil nutrients, as well as the test crop can also affect the establishment and performance of EM (Bajwa et al.,

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1999; Javaid et al., 2002; Javaid, 2010). In a 4-year field experiment under organic management (2003–2006) in Zurich, Switzerland, application of EM did not improve yields and soil quality (Mayer et al., 2010).

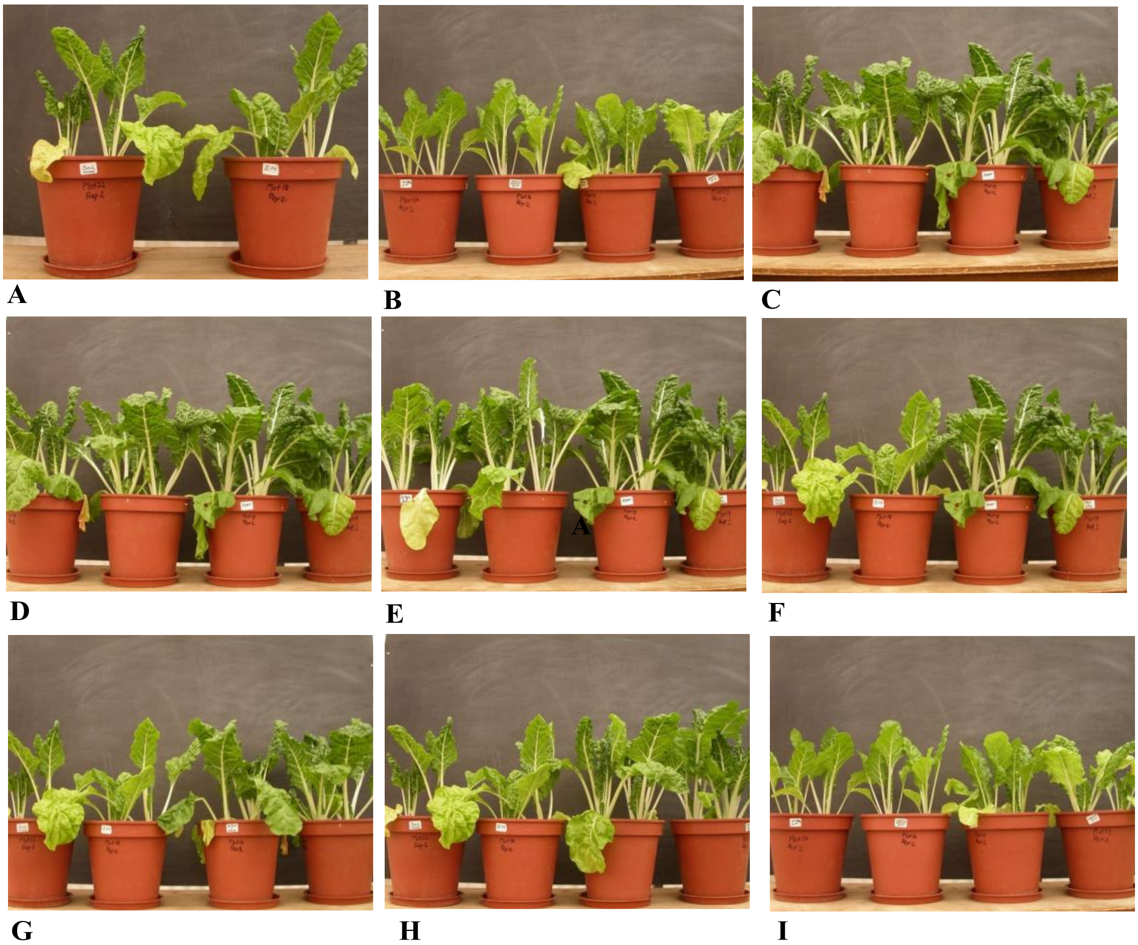

Figure 2. *Swiss chard plants growing in the greenhouse*
A *= Control and EM,* ***B*** *= ½ Reference Fertilizer + GM + EM, GM + EM and Comp + EM,* ***C*** *= GM, GM+ EM, Reference Fertilizer and Reference Fertilizer + EM,* ***D*** *= ½ Reference Fertilizer + Comp, ½ Reference Fertilizer + GM, Reference Fertilizer and Control,* ***E*** *= ½ Reference Fertilizer + GM, GM, Reference Fertilizer and Control,* ***F*** *= Control, EM, Reference Fertilizer and Reference Fertilizer + EM,* ***G*** *= Control, EM, ½ Reference Fertilizer + Comp, ½ Reference Fertilizer + Comp + EM,* ***H*** *= Control, EM, GM and Comp,* ***I*** *= GM, GM + EM, Comp, Comp + EM*

The application of the reference fertilisers (T3 and T4) caused a significant increase in yield for both the first and the second harvests (*Figure 3*), possibly due to the immediate release of nutrients from the added reference fertiliser. The application of T7, T8, T11 and T12 resulted in yield that was equivalent to that obtained with the application of the reference fertiliser (T3 and T4). These results indicate that half of the required mineral fertilisers can substitute half of the required fertiliser amount, possibly because of greater amounts of N and P supplied to the soil. Similar results were obtained by Khaliq et al. (2006), where the application of OM + EM + 1/2 mineral NPK

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yielded 2 091 kg ha^{-1} of seed compared to 2 165 kg ha^{-1} obtained from the recommended mineral NPK.

It is noteworthy that yield obtained from the control and treatments other than goat manure declined in the second harvest (*Figure 3*). This could be attributed to a decline in nutrient content as a result of nutrient removal by the first crop. The removal of nutrients by the first crop is confirmed by higher levels of leaf N content and subsequent N uptake by the first crop compared to the second crop (*Table 2*). Leaf P content increased during the second cropping and this ruled out the possibility of P limiting plant growth during the second cropping. These results are similar to those obtained by Tanner and Mugwira (1984). In their study, the application of manure to soil resulted in an increase in nutrient uptake by the second crop rather than the immediate crop. The implication of these results is that farmers should take measures to ensure that nutrients in organic material become available before plants begin their rapid development. Similar results were also obtained by Zhao et al. (2014).

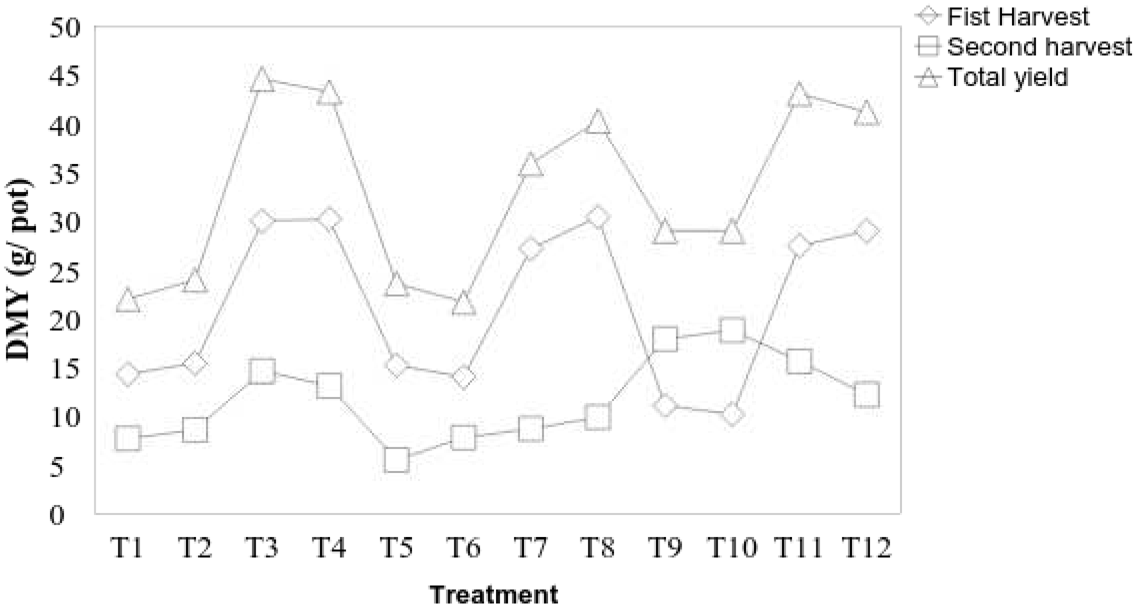

Figure 3. *Effects of amendments on Swiss chard dry matter yield (DMY)*
T1*: control,* ***T2****: effective microorganisms,* ***T3****: reference fertiliser,* ***T4****: reference fertiliser + EM,* ***T5****: compost alone,* ***T6****: compost + effective microorganisms,* ***T7****: half reference fertiliser + compost,* ***T8****: half reference fertiliser + compost + effective microorganisms,* ***T9****: goat manure alone,* ***T10****: goat manure + effective microorganisms,* ***T11****: half reference fertiliser + goat manure,* ***T12****: goat manure + half reference fertiliser + effective microorganisms*

Effects of amendments on leaf nutrient content

Leaf nutrient contents is a good predictor of plant responses to nutrients supplied through soil applied fertilisers. Nutrient management in organically managed soils is fundamentally different to soils managed conventionally, however, the underlying processes supporting soil fertility are not. This suggests that, although soil fertility is theoretically better in organic management, if there is a balanced supply of nutrients, i.e. there are no deficiencies or excesses of a nutrient that cause excessive consumption, the plant will take the nutrients in proportion to their needs (Herencia and Maqueda, 2016).

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There were significant ($p \leq 0.05$) treatment effects on leaf N content of both harvests (*Table 2*). Leaf N content ranged from 1.34 g kg^{-1} to 3.79 g kg^{-1} in the first harvest and 1.12 to 1.73 g kg^{-1} in the second harvest. The application of reference fertiliser significantly increased leaf N content of both the first and the second harvests as compared to the control. The highest leaf N concentration was observed with respect to the application of the reference fertiliser with EM in both the first and the second harvests. The application of half the reference fertiliser with compost resulted in a significant increase (18%) in leaf N content as compared to the control in the first harvest. Nitrogen is a critical component of proteins and biomolecules such as chlorophyll in plants (Fu et al., 2017). A similar trend was observed with respect to leaf N uptake, with the highest leaf N uptake being observed with reference fertiliser + EM treatment in both harvests. Due to the close relationship between N and protein, a similar trend was observed with crude protein in both harvests.

Leaf P content ranged from 0.06 g kg^{-1} to 0.14 g kg^{-1} in the first harvest and from 0.10 g kg^{-1} to 0.25 g kg^{-1} in the second harvest. Leaf P content remarkably increased from a depressed state in the first harvest to a significantly higher figure in the second harvest. The application of goat manure caused a significant ($p \leq 0.05$) increase in leaf P content relative to the control treatment (*Table 2*). It is known that with the addition of organic nutrients, the supply and availability of P is high in soils (Herencia et al., 2008). Manure application rates are routinely based on crop N requirements, which might have resulted in more P than is needed by the crop and this can cause a buildup of P in the soil (Edmeades, 2003) and consequently in plants. The P/N relationships for adequate crop development are usually 1/10 (Herencia and Maqueda, 2016), however, in the goat manure used in the current study, the P/N relationship was about 1/21.9. This amount would be in excess of the crop's needs. The application of goat manure with EM resulted in leaf P content that was significantly lower than that of the control.

Effects of EM, goat manure, compost and mineral fertiliser on selected soil properties

Post-cropping soil pH was significantly ($p \leq 0.05$) affected by different soil amendments (*Table 3*). The application of the reference fertiliser significantly depressed post-cropping pH, which declined to levels that were moderately acidic (*Table 3*). A similar trend was observed with the application of half reference fertiliser with compost (T7) and half reference fertiliser with compost and EM (T8).

The application of goat manure alone (T9) or with half the reference fertiliser (T11), without or with EM (T12), significantly increased post-cropping soil pH relative to the control treatment. The highest post-cropping pH was observed with the sole application of goat manure (T9). These results indicate that goat manure has a liming effect, whereas the mineral fertiliser has acidifying effects. The liming effects of goat manure can be of great value in areas like the Eastern Cape, South Africa, parts of which have critically low soil pH (Mandiringana et al., 2005). Similar results were obtained by Mhlontlo et al. (2007), in whose study the application of sheep kraal manure at rates greater than 2.5 t ha^{-1} resulted in higher pH values compared to the control and the mineral fertiliser treatments.

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Table 2. *Effects of amendments on leaf N content, N uptake, crude protein, P, and K in Swiss chard plants*

Treatments	**First harvest**					**Second harvest**				
	N (g kg^{-1})	N uptake (mg pot^{-1})	Crude protein (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})	N (g kg^{-1})	N uptake (mg pot^{-1})	Crude Protein (g kg^{-1})	P (g kg^{-1})	K (g kg^{-1})
T1	2.3^{bc}	477.8^{ecd}	0.59^{bc}	0.007^{bc}	1.24^{ab}	1.46^{ac}	150.0^{ab}	0.15^{bc}	0.25^{a}	0.89^{ab}
T2	1.3^{4c}	200.0^{e}	0.33^{c}	0.09^{abc}	1.15^{ab}	1.52^{ab}	223.35^{a}	0.08^{c}	0.22^{ab}	0.80^{b}
T3	3.0^{ab}	876.7^{ab}	0.75^{ab}	0.06^{c}	1.05^{b}	1.37^{cbd}	140.92^{ab}	0.19^{ab}	0.17^{bc}	0.84^{ab}
T4	3.8^{a}	1120.2^{a}	0.95^{a}	0.06^{c}	1.04^{b}	1.73^{a}	177.83^{ab}	0.24^{a}	0.21^{ab}	0.90^{ab}
T5	1.6^{c}	2389^{ed}	0.40^{c}	0.08^{abc}	1.15^{ab}	1.26^{cbd}	132.14^{ab}	0.10^{c}	0.24^{a}	0.90^{ab}
T6	1.4^{c}	192.5^{e}	0.35^{c}	0.07b^{c}	1.31^{ab}	1.29^{cbd}	152.36^{ab}	0.09^{c}	0.22^{ab}	1.04^{ab}
T7	2.8^{ab}	757.0^{bc}	0.69^{ab}	0.06^{c}	1.31^{ab}	1.30^{cbd}	203.14^{ab}	0.17^{ab}	0.25^{a}	0.79^{b}
T8	2.9^{ab}	728.4^{bc}	0.71^{ab}	0.13^{ab}	1.21^{ab}	139^{cbd}	171.67^{ab}	0.18^{ab}	0.25^{a}	0.77^{b}
T9	1.6^{c}	180.8^{e}	0.41^{c}	0.10^{abc}	1.25^{ab}	1.12^{d}	157.34^{ab}	0.10^{c}	0.0^{d}	0.11^{a}
T10	2.0b^{c}	199.8^{e}	0.50^{bc}	0.14^{a}	1.27^{ab}	1.33^{cbd}	120.18^{b}	0.12^{cb}	0.11^{cd}	0.91^{ab}
T11	1.7^{c}	482.3^{ecd}	0.42^{c}	0.07^{bc}	1.21^{ab}	1.26^{cbd}	158.46^{ab}	0.10^{c}	0.19^{ab}	1.03^{ab}
T12	2.0^{bc}	560.8^{bcd}	0.49^{bc}	0.06^{c}	1.39^{a}	1.23^{cd}	131.27^{ab}	0.12^{bc}	0.21^{ab}	0.91^{ab}
C.V	32.64	47.924	32.64	47.53	18.09	14.21	38.64	32.64	23.02	22.10

T1: control, **T2**: effective microorganisms, **T3**: reference fertiliser, **T4**: reference fertiliser + EM, **T5**: compost alone, **T6**: compost + effective microorganisms, **T7**: half reference fertiliser + compost, **T8**: half reference fertiliser + compost + effective microorganisms, **T9**: goat manure alone, **T10**: goat manure + effective microorganisms, **T11**: half reference fertiliser + goat manure, **T12**: goat manure + half reference fertiliser + effective microorganisms
Means in a column with a different letter are statistically different (LSD test; $p \leq 0.05$).

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***Table 3.** The effects of amendments on selected soil properties after harvest of Swiss chard*

Treatment	pH (1:2.5 soil: water)	EC (µS/cm)	N (g kg^{-1})	Total C (g kg^{-1})	C:N	P (g kg^{-1})	K (g kg^{-1})
T1	5.7^{c}	9.7^{c}	0.5^{e}	7.4^{c}	13.2^{abc}	0.4^{g}	2.6^{d}
T2	5.7 ^{c}	10.6^{c}	0.6cd^{e}	7.7^{c}	14.8^{a}	0.4^{g}	3.3^{cd}
T3	5.2^{e}	48.6^{a}	0.8^{ab}	7.8^{c}	14.8^{a}	1.1^{a}	2.8^{d}
T4	5.1^{e}	44.4^{a}	0.6^{cde}	8.1^{bc}	12.7^{abc}	1.0^{ab}	2.0^{d}
T5	5.5^{cd}	10.1^{c}	0.6^{cde}	8.3^{bc}	13.6^{abc}	0.4^{g}	3.1^{d}
T6	5.5^{cd}	10.1^{c}	0.5^{e}	7.7^{c}	14.1^{ab}	0.4^{g}	3.0^{d}
T7	5.2^{e}	28.7^{b}	0.7^{bcde}	8.6^{bc}	11.8^{bc}	0.8^{cd}	2.8^{d}
T8	5.3^{de}	27.^{3b}	0.8^{ab}	10.2^{a}	11.4^{c}	0.7^{de}	2.0^{d}
T9	6.5^{a}	32.2^{b}	0.8^{ab}	10.3^{a}	12.1^{bc}	0.6^{ef}	5.2^{abc}
T10	6.2^{b}	22.8^{b}	0.9^{a}	10.4^{a}	11.4^{c}	0.5^{fg}	6.3^{a}
T11	6.1^{b}	31.1^{b}	0.7^{bcde}	9.4^{ab}	14.2^{ab}	0.8^{cd}	3.9^{bcd}
T12	6.0^{b}	45.1^{a}	0.8^{ab}	10.0^{a}	13.4^{abc}	0.9^{bc}	5.5^{abc}
C.V	3.4	29.3	16.77	11.46	13.2	15.3	40.9

T1: control, T2: effective microorganisms, **T3;** reference fertiliser, **T4**; reference fertiliser + EM, **T5;** compost alone, **T6**: compost + effective microorganisms, **T7**: half reference fertiliser + compost, **T8**: half reference fertiliser + compost + effective microorganisms, **T9**: goat manure alone, **T10**: goat manure + effective microorganisms, **T11;** half reference fertiliser + goat manure, **T12;** goat manure + half reference fertiliser + effective microorganisms
Means in a column with a different letter are statistically different (LSD test; $p \leq 0.05$)

The highest EC value was observed in plots where the reference fertiliser was applied. A similar pattern was observed in respect of the application of sole goat manure or goat manure in combination with half the reference fertiliser, with or without EM.

There were significant ($p \leq 0.05$) treatment effects on residual soil N concentration (*Table 4*), although the values did not significantly differ from the initial concentration. Soil N levels associated with the reference fertiliser, half the reference fertiliser + compost + EM, goat manure, goat manure + EM, half the reference fertiliser + goat manure, and half the reference fertiliser + goat manure + EM were significantly higher than those of the control treatment, suggesting that the plants did not exhaust N from these added amendments (*Table 3*). The increase in N that was observed could possibly be due to the slow release of nutrients through mineralisation from these organic materials. The application of goat manure + EM resulted in the highest soil N and C contents, suggesting that EM increased the mineralisation of goat manure applied to the soil.

A similar trend was observed with soil C, and the greatest amounts of soil N and C were observed in soils treated with goat manure and EM together. The observed decrease in the soil C:N ratio indicated a build-up of the N pool in the soil. The application of different amendments decreased extractable soil P to below initial levels. The highest extractable P was observed in respect of the application of reference fertiliser, suggesting that the crop had not exhausted the soil P from the fertiliser that had been applied.

Soil residual K in pots treated with goat manure, goat manure + EM and half the reference fertiliser + goat manure, and EM was significantly higher than that of the control treatment. The highest soil residual K was observed in pots where goat manure

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was applied with EM, although the manure used had a relatively low concentration of K. Kraal manure has, on average, about 2% K, which is far higher than the 0.5 % contained in the goat manure used (Bornman et al. 1989; Kizzar et al., 2010; Materechera and Mkhabela, 2016). General residual soil K in all the amendments exceeded the critical level of 80–120 mg kg^{-1} suggested by Bornman et al. (1989). Results from this study are consistent with what was reported by Laker (1976) and Ayanda et al. (2016), namely, that most South African soils are not deficient in K.

Conclusion

EM application had inconsistent effects on Swiss chard yields. The use of goat manure with a narrower C:N ratio than the compost used in earlier studies did not improve EM effectiveness, indicating that the observed ineffectiveness of EM was not related to the quality of the organic material used. However, the results of this study show the benefits of the combined application of organic amendments with half of the recommended mineral fertiliser over the separate full application of inorganic fertiliser or organic amendment.

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FOOD CONSUMPTION PATTERNS OF SUB-SAHARAN AFRICAN IMMIGRANTS RESIDING IN GAUTENG PROVINCE, SOUTH AFRICA

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Abstract. Migration often leads to the loss of what is known as the "healthy immigrant effect" due to the adoption of unhealthy eating habits upon resettlement in host countries. A descriptive study was conducted to assess the current food practices of sub-Saharan immigrants residing in Gauteng Province, South Africa. Questionnaires were administered to 194 female participants residing in Gauteng. Data relating to socioeconomic factors, the adoption of South African food culture in general and specific food items that have been adopted were collected. The association between food adoption and socioeconomic factors was tested. Among the households polled, 78.4% had adopted South African food to varying degrees. Four adoption patterns emerged: strict continuity with traditional food; very limited adoption; limited adoption; and complete adoption of South African food culture. With regard to the association between food adoption and factors tested, the region of origin (p<0.000) was highly significant, while money spent on food was marginally significant (p<0.077). Dietary acculturation was characterised by a high intake of energy-dense, high-fat and low-micronutrient foods. Culturally appropriate nutrition education studies are required. Future studies should assess the impact of these dietary patterns on health outcomes among immigrants living in South Africa.
Keywords: *dietary acculturation, dietary patterns, food culture, food adoption patterns*

Introduction and background

Recent immigration trends indicate that 70% of all international migration is interregional and takes place within the African continent (Adepoju, 2008). Consequently, ever since the apartheid dispensation ended in 1994, South Africa has seen an increase in the number of sub-Saharan immigrants due to unstable economic conditions, soaring ethnic conflicts, volatile political situations and drought situations in most African countries (Njomo, 2013; Statistics South Africa, 2014a). However, research has shown that immigrants undergo dietary acculturation upon settlement in new countries (Garnweidner et al., 2012; Njomo, 2013) due to unavailability and inaccessibility of their traditional ingredients in the host countries (Garnweidner et al., 2012; Deng et al., 2013). Dietary acculturation is defined as the process by which immigrant minority groups adopt the dietary habits of the host society (Deng et al., 2013).

Dietary acculturation often results in the adoption of unhealthy eating habits (Okafor et al., 2014; Terragni et al., 2014) and subsequent loss of what is known as the "healthy immigrant effect" (HIE) (Sanou et al., 2014). HIE is defined as a situation whereby immigrants are usually healthier than native-born population when they first arrive in host countries due to medical screening they are subjected to as part of the selection

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processes (Sanou et al., 2014). However, upon settlement in host countries, previous studies indicate (Lesser et al., 2014; Sanou et al., 2014) that immigrants loose the Healthy Immigrants Effect due to, among other factors dietary acculturation. Evidence suggests that African immigrants are at risk of weight gain and poor health outcomes owing to their poor food choices, when compared with immigrants from other continents (Venters and Gany, 2011; Okafor et al., 2014). According to Venters and Gany (2011), this could explain why the leading chronic diseases among African immigrants are hypertension and diabetes, which are lifestyle diseases.

It is reported that immigrants tend to increase their intake of ready-to-eat processed foods because they are unfamiliar with new foods and cooking items (Deng et al., 2013). According to a study that was conducted among Somali refugees in the United States of America (USA), the intake of snacks and sugary beverages increased significantly in this group of immigrants upon resettlement (Dharod et al., 2011). Replacing high-nutritive food with high-fat and calorie-dense food was also reported in a study that was conducted among Latin American immigrants residing in Toronto (Vahabi and Damba, 2013). Higher dietary acculturation characterised by increased consumption of fast foods and meat and a decrease in the consumption of fruits and vegetables was reported in a study that was conducted in the USA among African immigrants (Okafor et al., 2014). This places immigrants at a higher risk of developing lifestyle diseases – a situation which, if left unattended, could place an enormous burden on the health system of the host country. Sanou et al. (2014), in a review of acculturation studies conducted among immigrants living in Canada, argue that the burden of lifestyle diseases increases consistently after immigrants' settlement in host countries.

Although most studies on dietary acculturation have been conducted on immigrants moving from developing to developed countries, an increase in the intake of sweets, sweet baked goods and sweetened fruit drinks was reported in a study on Liberian immigrants residing in Ghana (Ross et al., 2016). This suggests that immigrants moving from one developing country to another are not immune to these unhealthy dietary changes when settling in developing host countries. Two past studies conducted in South Africa showed that sub-Saharan immigrants consume their indigenous foods at least three times a week (Njomo, 2012, 2013). The question, then, is what happens during the others days of the week? It is possible that some level of dietary acculturation is taking place among this group. Furthermore, what could be the possible implications of this dietary acculturation on the “Health Immigrant Effect”? However, there is no evidence of studies that have investigated dietary acculturation or factors that affect dietary acculturation among sub-Saharan immigrants living in South Africa.

The authors of this paper had three objectives: (1) to assess the dietary patterns of sub-Saharan immigrants residing in Gauteng; (2) to identify patterns of dietary acculturation among sub-Saharan immigrants upon resettlement; and (3) to identify factors that significantly affect the adoption of South African food.

Methods

Research design

A descriptive survey study design was adopted to obtain responses that would fulfil the objectives of this study. The study was conducted in Tshwane and Johannesburg

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because these two metropolitan municipalities together host the largest number of immigrants (Landau and Gindrey, 2008; Njomo, 2013).

Study area

South Africa does not have statistics on all immigrants currently residing in the country (Statistics South Africa, 2013). Therefore, information on the number of permits that were issued by the Department of Home Affairs in 2011 and 2013 was considered (Statistics South Africa, 2013, 2014a) when determining the regions to be included in this study. According to Statistics South Africa (2016), the majority of immigrants are from Southern Africa, followed by Central Africa and then East Africa. Therefore, for the purposes of this study, only immigrants from these regions were considered for this study.

Development of questionnaire

Literature from previous research informed the objectives of the study, which were used to develop the survey questionnaire. Gaps in the literature indicated the need to gain a greater understanding of the dietary patterns, acculturation and factors that affect dietary acculturation of sub-Saharan immigrants in South Africa. For the purposes of clarity, relevance, language and cultural appropriateness, the first draft of the questionnaire was reviewed independently by four sub-Saharan immigrant academics that have been residing in the country for over 15 years. Feedback was discussed by all authors to reach consensus before changes were effected.

The following changes were introduced, based on the recommendations of the reviewers: An option for self-employment was added; categories of income levels were created, rather than making this question open-ended; possible reasons for wanting to continue with the traditional food culture were added, from which respondents could choose; the wording of some questions was revised to make them more relevant to the target community (e.g. the term “food safety” was deemed to be unclear and was therefore changed to “quality of the food”); and owing to the diverse origin of immigrants, the question on the country of origin was changed to region of origin. The final draft questionnaire was then circulated to a group of 20 academics for final review.

The questionnaire was piloted on 34 female participants representing households from Southern, Central, East and West African regions. Two major changes were made, based on the results of the pilot study. Among the Southern African immigrants, the highest number of immigrants were from Zimbabwe, accounting for approximately 42.2% of the permits that were issued (Statistics South Africa, 2013, 2014b, 2016). During the pilot study, it was discovered that there was not much difference between South African and Zimbabwean food culture; therefore, the Southern African region was dropped from the main study. The question about the immigration status was also excluded, as most respondents were not comfortable answering that question. The final questionnaire had 4 sections aimed at capturing data on: socio-demographic and economic details of the respondents, adoption of the South African food, food items that have been adopted since settled in South Africa and 3-day food recall.

Selection of participants

The study targeted women, who are habitually responsible for food preparation in most households in African societies (Garnweidner et al., 2012; Njomo, 2013). In this

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study, owing to the lack of a sampling frame, it was not possible to adopt random sampling, which is known to yield representative samples. Furthermore, since relatively little is known about the phenomenon under investigation, snowball sampling was considered the most appropriate sampling technique for the study. Churches that host highest numbers of immigrants, businesses that employ immigrants, personal contacts of data collectors, referrals from academic staff members and networking were used to identify and recruit the first six participants of the study (two from each region). Each participant was then requested to identify two other immigrant households. This process was repeated until a statistically significant number of participants was reached. One hundred and ninety-four (n=194) female participants representing households from Central, East and West African regions were identified and invited to participate in the study.

Data collection

Four university students of sub-Saharan African descent were recruited and trained as data collectors. The data were collected between August 2015 and January 2016. A structured questionnaire consisting of both closed and open-ended questions was used to gather data on socio-demographic characteristics, dietary patterns and dietary acculturation. A three-day food recall was also used at the end of each questionnaire to triangulate the information gathered on the food items that have been adopted since the immigrants' arrival in South Africa. In cases where the responses were deemed insufficient or unclear, probing questions were used to elicit more information.

Data analysis

Quantitative data were coded and entered into a Microsoft Excel® worksheet. The data were then checked for any inconsistencies and missing values before analysis was performed using the statistical package IBM SPSS version 23 (2015). Data were summarised and descriptive statistics presented as graphs and tables. The probit model was used to identify associations between the adoption of South African food and socio-demographic and economic factors of the households. In this study, households that had adopted South African food culture were coded as 1, whereas those that had not adopted the South African food culture were denoted as 0.

Assumption of general probit model: $Y = \Pr(Y=1 \mid X) = \Phi(X' \beta)$,

where Pr denotes probability; Φ is the cumulative distribution function (CDF) and β is the parameters

The probit model, as a latent variable model with an auxiliary random variable, is expressed as: $Y^{*} = \beta' X + \varepsilon$,

where $\varepsilon \sim N(0,1)$. Then Y can be viewed as an indicator of whether this latent variable is positive:

$$Y = 1_{\{Y^{*}>0\}} = \begin{cases} 1 \text{ if } Y^{*} > 0 \text{ i.e. } -\varepsilon < X'\beta, \\ 0 \text{ otherwise} \end{cases} \quad \text{(Eq. 1)}$$

Specific regression model: $ASFC = \beta_0 + \beta_1 A + \beta_2 G + \beta_3 Edc + \beta_4 B + \beta 5DR + \beta_6 P + \beta_7 MS + \beta_8 R + \beta_9 ES + \beta_{10} Y + \beta_{11} MSF$

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$$Y = \begin{cases} 1\ if\ households\ have\ adopted\ South\ African\ food\ culture \\ 0\ if\ households\ have\ not\ adopted\ South\ African\ food\ culture \end{cases}$$

Variable code	Name of variable	Variable description	Unit of measurement
Dependent variable			
Y = ASFC	Adoption of South African food culture	1 if households have adopted South African food culture 0 if households have not adopted South African food culture	Dummy
Independent variables			
X_1 = A	Age	Age of household member	Number
X_3 = EdcL	Education level	1 if respondent has no formal school education 2 if respondent has primary education 3 if respondent has secondary education 4 if respondent has tertiary education	Dummy
X_4 = DR	Duration of residence	1 if respondent has resided here for less than a year 2 if respondent has resided here for 1–3 years 3 if respondent has resided here for 4–6 years 4 if respondent has resided here for more than 7 years.	Dummy
X_5 = MS	Marital status	1 if respondent is married 2 if respondent is unmarried 3 if respondent is divorced 4 if respondent is widowed	Dummy
Variable code	**Name of variable**	**Variable description**	**Unit of measurement**
X_6 = R	Region of origin	1 if respondent is from West Africa 2 if respondent is from East Africa 3 if respondent is from Central Africa	Dummy
X_7 = ES	Employment status	1 if respondent has a full-time job 2 If respondent has a part-time job 3 If respondent has a temporary assignment 4 if respondent is self- employed 5 if respondent is a student 6 if respondent is unemployed	Dummy
X_8 = Y	Household income	0 if there was no response 1 if respondent earns under R5 000 2 if respondent earns between	Rand

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		R5 000 and R10 000 3 if respondent earns between R11 000 and R15 000 4 if respondent earns between R16 000 and R20 000 5 if respondent earns between R21 000 and R25 000 6 if respondent earns between R26 000 and R30 000 7 if respondent earns more than R30 000	
X_9 =MSF	Money spent on food	1 if respondent spends under R500 2 if respondent spends between R500 and R1 499 3 if respondent spends between R1 500 and R2 499 4 if respondent spends between R2 500 and R3 499 5 if respondent spends between R3 500 and R4 500	Dummy

Ethical considerations

Ethical clearance (2014/CAES/113) for the study was obtained from the College of Agriculture and Environmental Science, University of South Africa before the study could commence. After study objectives were explained to the participants, only those who granted informed consent participated in the study.

Results

Socioeconomic results

As shown in *Table 1*, the majority of the respondents (79%; n=153) were aged between 20 and 39 years. Most respondents (53.6%; n=104) were single and 56.7% (n=110) had tertiary education, while 43.3% had only a high school education or lower. More than half (62.9%; n=122) of the respondents had lived in South Africa for more than four years. Most respondents (41%; n=80) were from West Africa, while 30% (n=58) were from Central Africa and 29% (n=56) were from East Africa.

***Table 1.** The socio-demographic details of respondents showing their age, education, marital status and duration of residence in South Africa and region of origin*

Variable	Number of respondents (n=194)	Percentage (%)
Age		
Below 20	7	3.6
20–29	89	46
30–39	64	33
40–49	24	12.4
50–59	8	4.0

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60–69	1	0.5
Over 70 years	1	0.5
Educational level		
No formal education	6	3.1
Less than high school education	78	40.2
Tertiary education	110	56.7
Marital status		
Single	104	53.6
Married	81	41.7
Divorced/widowed	9	4.6
Duration of residence in South Africa		
Less than a year	10	5.1
1–3 years	62	32.0
4–6 years	60	30.9
7 years or more	62	32.0
Region of origin		
West Africa	80	41
East Africa	56	29
Central Africa	58	30

Table 2 describes employment status, household income and money spent on food, as reported by the households included in the study. Monthly household income of the respondents ranged from R500 to R30 000, with 32.0% (n=62) earning under R5 000 and only 16.5% (n=32) earning above R20 000.

Table 2. *Economic details of the respondents*

Variable	Number of respondents (n=194)	Percentage (%)
Employment status		
Full-time job	61	31.4
Part-time job	18	9.3
Temporary job	3	1.5
Self-employed	80	41.2
Student	13	6.7
Unemployed	18	9.2
Household income per month		
No response	3	1.5
Under R5 000	62	32.0
R5 000–R10 000	56	28.9

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R11 000–R15 000	27	13.9
R16 000–R20 000	14	7.2
R21 000–R25 000	5	2.6
R26 000–R30 000	12	6.2
Above R30 000	15	7.7
Money spent on food per month		
Under R500	15	7.7
R500–R1 499	65	33.5
R1 500–R2 499	43	22.1
R2 500–R3 499	45	23.1
R3 500–R4 500	26	13.4

Adoption of the South African food culture

Out of 194 respondents, 78.4 % (n=152) indicated that they had adopted the South African food culture (*Table 3*). Among those who had adopted South African foods, the level of adoption varied considerably. Based on the findings of this study, the following four (4) patterns of dietary acculturation emerged: strict continuity with their traditional food (21.6%; n=42); very limited adoption (21.1%; n=39); limited adoption (50.5%; n=98); and complete adoption of South African food culture (7.7%; n=15). Complete adoption of South African food was explained as eating South African food during all mealtimes and eating traditional food only on special occasions, such as at cultural gatherings, weddings and naming ceremonies. Limited adoption meant eating South African food in combination with traditional food; very limited adoption was used to describe eating traditional food at all mealtimes, while eating South African foods only when visiting friends or dining out. Strict continuity with traditional food meant that the respondents always eat traditional food for all meals.

Table 3. *Adoption of South African food*

Variable	Number of respondents (n=194)	Percentage (%)
Adoption of South African food		
Yes	152	78.4
No	42	21.6
Level of adoption		
Strict continuity with traditional food	42	21.6
Limited adoption	98	50.5
Very limited adoption	39	20.1
Strictly SA food	15	7.7
Reasons for adopting		
Not applicable	42	21.6

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Traditional food too expensive	76	39.2
Unavailability of traditional food	49	25.3
Convenience	15	7.7
I like South African food	8	4.1
My kids prefer it	4	2.1
Reasons for not adopting		
Not applicable	152	78.4
Unfamiliar taste	33	17.0
Inability to cook SA food	6	3.1
SA food is unhealthy	3	1.5

During the interviews, it became clear that immigrants did not distinguish food that was part of the South African cuisine from globalised or westernised foods. They considered all food – including fast food – to be part of the traditional South African food culture, as long as it fell outside their original food culture.

Respondents gave various reasons for adopting South African food (see *Table 3*). Among the households that reported adopting the South African food culture, the most common reason was that the price of traditional food was too high (50%; n=76), followed by the unavailability of the traditional food (32.2%; n=49).

Of the 42 respondents that have not adopted South African food, the main reason given was the unfamiliar taste (79%; n=33). Other reasons included inability to cook South African food (14.2%; n=6) and the fact that other immigrants believed the food is unhealthy (7%; n=3).

Food items that have been adopted since resettlement

An assessment of the food items that the respondents had started eating after settling in South Africa revealed the following to be the most common: pap (84.5%; n=164); fried potato chips (43.8%; n=85); cold drinks (42.8%; n=83), fast foods (37.6%;n=73); *sphathlo* (30.9%; n=60); *vetkoek* (fat cakes) (30.4%; n=59); and spinach (24.2%;n=47). Pap is a typical South African staple food made from maize meal, while *sphathlo* (sometimes known as *kota*) is an African burger made of a quarter loaf of bread and usually filled with at least five ingredients, including potato chips, cheese, polony, Russians (spicy sausages), atchar (a spicy condiment), fried eggs and tomato sauce. Other items that are sometimes also included in *sphathlo* are chakalaka (a spicy vegetable relish), braai meat, samp (coarsely ground mealie meal) and beans. *Sphathlo* is commonly consumed in the townships of Gauteng.

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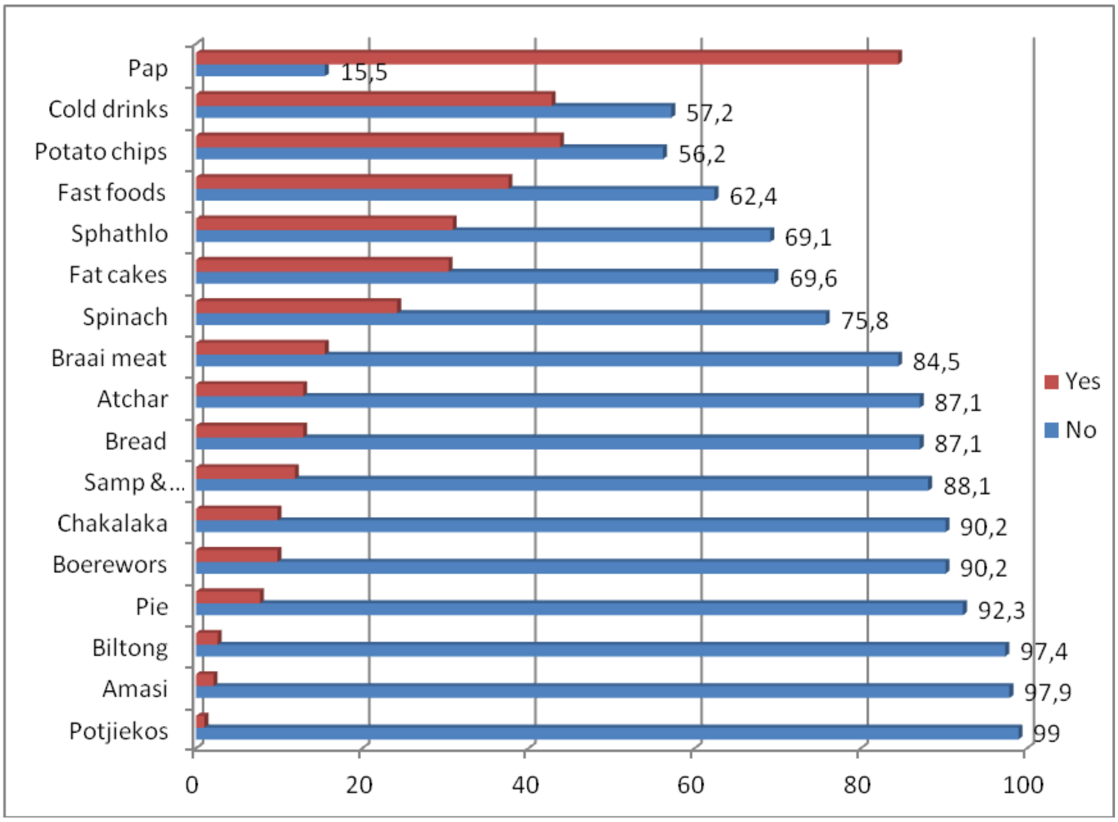

Figure 1. *South African food adoption showing different food items that have been adopted by immigrants since their re-settlement in South Africa*

Consumption of fizzy drinks was also common among immigrants, with most respondents consuming at least one fizzy drink every day. The leading fast food outlets mentioned by respondents were Kentucky Fried Chicken, MacDonald’s, Steers, Nando's and Roman's Pizza.

Factors associated with the adoption of South African food

At 0.05 cut off significance level, the region of origin was highly significant ($p<0.000$), while money spent on food was marginally significant ($p<0.077$). No statistically significant associations were observed for the rest of the factors that were tested.

Table 4. *Factors associated with the adoption of south african food*

Parameter estimates							
						95% confidence interval	
	Parameter	Estimate	Std. error	Z	Sig.	Lower bound	Upper bound
PROBIT[a]	Region	-.264	.038	-6.920	.000	-.338	-.189
	Marital status	-.057	.043	-1.338	.181	-.142	.027
	Age	-.013	.033	-.386	.700	-.077	.052
	Qualification	.007	.025	.289	.773	-.042	.056

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	Employment status	-.011	.019	-.551	.582	-.049	.027
	Household income	.008	.018	.433	.665	-.027	.043
	Money spent on food	-.027	.015	-1.770	.077	-.057	.003
	Duration of residence	-.013	.034	-.378	.705	-.079	.053
	Intercept	-1.571	.245	-6.416	.000	-1.815	-1.326

a. PROBIT model: PROBIT(p) = Intercept + BX

Discussion

Results on the age of the respondents confirm findings that the majority of the immigrants are young (Vahabi and Damba, 2013; Anderson et al., 2014) and have probably moved in search of better economic conditions. While most immigrants participating in our study were fairly educated (56.7%; n=110), the low-income categories (i.e. below ZAR15 000 per month) into which the majority of respondents (74.8%; n=145) fell suggest that our study population falls into the low-income bracket. This income is below the South African average monthly income of ZAR17 517(Statistics South Africa, 2016). Low incomes and the fact that only 31.4% of immigrants are in full-time jobs suggest that they are finding it difficult to secure decent jobs. The prevalence of low socioeconomic status among the respondents in this study was consistent with the results of similar studies of sub-Saharan immigrants that have resettled in other countries (Vahabi and Damba, 2013; Anderson et al., 2014; Ross et al., 2016). As a result, their dietary acculturation is likely to be characterised by negative dietary changes (Kiptinness et al., 2011). Difficulty in finding employment among well-educated immigrants was associated with language barriers (Vahabi and Damba, 2013) and employment restrictions imposed on foreign nationals (Shackelford, 2010). In the current study, the low employment level could be aggravated by the high unemployment rate in South Africa, which is currently at 26.6% (Statistics South Africa, 2016).

Consistent with previous studies, the current study revealed that even though the majority of immigrants try to preserve their traditional food culture upon resettlement, some level of dietary acculturation takes place (Garnweidner et al., 2012; Njomo, 2013; Sanou et al., 2014). For example, in a Norwegian study on African and Asian immigrants conducted by Garnweidner et al. (2012), different patterns of dietary acculturation were observed. Therefore, the authors of the current study are of the view that dietary acculturation does not move in a linear process. This view is supported by Deng et al. (2013), who contends that Chinese immigrants residing in North America do not just change their diets from traditional to strictly new foods upon resettlement. Instead, immigrants retain traditional foods, find new ways of incorporating their traditional ingredients and exclude others, while adopting new ones (Satia, 2010).

While Garnweidner et al. (2012) identified three patterns of dietary acculturation, the authors of the current study observed four patterns. However, in the same study by Garnweidner et al. (2012), none of the participants had completely adopted the host country's food culture.

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In the case of South Africa, the fact that immigrants are able to continue with their traditional or ethnic diets could be attributed to traditional foods' being available and sold by small ethnic shops (Njomo, 2012). However, these ethnic shops are reportedly very expensive when compared with the mainstream supermarkets (Njomo, 2012; Dharod et al., 2013). This situation could lead to food inaccessibility and hence accelerated dietary acculturation.

As was observed in previous studies (Garnweidner et al., 2012; Deng et al., 2013), high prices and unavailability of traditional food were the main reasons given by respondents for adopting South African foods. In view of this, adopting the South African food culture suggests that dietary change is not voluntary and might happen haphazardly before immigrants get a chance to familiarise themselves with the local food culture. This has important policy implications because of evidence suggesting that most immigrants often replace pricey, nutrient-rich foods with cheaper, energy-dense but low-micronutrient foods (Anderson et al., 2014). According Deng et al. (2013), when dietary acculturation occurs, breakfast is the first meal to be replaced by food items such as oatmeal, milk, bagels and cream cheese, a transition that predisposes the consumers to obesity and type 2 diabetes. Although there are dietary guidelines for the general South African population (Vorster et al., 2013), there is no record of nutrition education programmes that target immigrants in South Africa. The authors are therefore of the view that welfare resettlement and nutrition education programmes are required to help minimise the negative impact of acculturation among immigrants settling in the country.

Studies show that taste is associated with resistance to adopting the new food culture (Garnweidner et al., 2012; Dharod et al., 2013; Vahabi and Damba, 2013). This is consistent with the findings of this study, which showed that unfamiliar taste contributed to some immigrants' not adopting South Africa’s food culture. A similar observation was made by Vahambi and Damba (2013) in a study they conducted in Toronto among Latin American immigrants. There, too, they concluded that difference in the taste of food resulted in a rejection of the new food culture.

In examining the changes in food habits, the results of our study indicated that the intake of energy-dense, highly processed food among immigrants had increased significantly after they had settled in South Africa. These findings are consistent with the findings of previous studies (Deng et al., 2013; Lesser et al., 2014; Okafor et al., 2014). Okafor et al. (2014) also reported that dietary changes among African immigrants in the United States were characterised by foods high in fat, sugar and cholesterol. An increase in the intake of convenience foods, fizzy drinks, desserts, candy and dining out among Asian immigrants residing in Canada was also reported by Lesser et al. (2014). Deng et al. (2013) argue that as Chinese immigrants settle in the United States, their diets tend to become more westernised. These dietary behaviours are highly discouraged as they are associated with lifestyle diseases such as obesity and type 2 diabetes (Deng et al., 2013; Okafor et al., 2014).

The unhealthy food choices observed among immigrants have been attributed to their low socioeconomic status. This is because these poor-quality food items are usually less costly than more nutritious food (Dharod et al., 2011; Okafor et al., 2014). In addition, Okafor et al. (2014) suggest that since most immigrants live below the poverty line in their new home countries, they are usually forced to work multiple low-paying jobs, thus reducing food accessibility and time to prepare traditional food dishes. In view of the fact that the current study also indicated that the majority of the immigrants (76.3%)

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live below the average monthly gross income of ZAR17 517(Statistics South Africa, 2016), they are likely to experience difficulty in accessing nutritious food, which tends to be more costly. Apart from economic constraints, the increase in the consumption of unhealthy fast foods could also be due to a lack of knowledge on how to prepare new food items. Meanwhile, unhealthy nutrition transitions have also been associated with lifestyle factors, such as urbanisation and globalisation, which result in the high accessibility of fast foods and fizzy drinks (Satia, 2010). Furthermore, it is reported that prior to immigration, most immigrants are accustomed to small shops, open markets and fresh foods (Njomo, 2012; Terragni et al., 2014); therefore, moving to a place like Johannesburg or Pretoria – where there are many large supermarkets – could curtail their shopping capabilities. This could explain why immigrants resort to fast, cheap, energy-dense foods.

It is not always the case that immigrants respond negatively when faced with the challenges of adopting new food cultures. Some authors have found that immigrants sometimes make positive dietary changes (Lesser et al., 2014; Sanou et al., 2014). The question, then, is what causes these immigrants to make positive changes, while others do not? Is it a lack of knowledge of nutrition and unfamiliarity with the cooking methods and new foods that prevent immigrants from making healthy food choices? Available evidence suggests that positive dietary changes are associated with nutrition education initiatives (Lesser et al., 2014).

Consistent with the observation by Okafor et al. (2014), the region of origin in the current study was highly significant (*P=.000)* at the confidence level of 5%. Okafor et al. (2014) observed in their study that immigrants from Ethiopia and Nigeria were more acculturated than immigrants from other sub-Saharan countries. Moreover, Okafor et al. (2014) observed that the diets of the more acculturated immigrants were characterised by an increased consumption of fast foods and a decreased consumption of fruits and vegetables.

The other factor that was marginally associated with the adoption of South African foods was the money spent on food. This was expected, given that the majority of immigrants (76.3%) who participated in this study indicated that they lived on less than the average national monthly gross income of South Africa, namely ZAR17 517(Statistics South Africa, 2016).

In previous studies, factors such as age, education level, income (Deng et al., 2013; Okafor et al., 2014) and the duration of residence (Dharod et al., 2011; Okafor et al., 2014) had a significant influence on food adoption, whereas in the current study these factors did not prove to be significantly associated with the adoption of South Africa food culture. The fact that there are differences between the current study and the studies reported on above in terms of geographical location of the studies and methodologies adopted could explain the differences in the findings.

In the literature, length of residence has been associated with dietary acculturation (Deng et al., 2013; Lesser et al., 2014). For example, Deng et al. (2013), observed that a longer duration of stay by Korean immigrants in the USA resulted in higher acculturation among younger immigrants (Deng et al., 2013). In contrast, length of residence was not associated with dietary acculturation in the current study. However, the findings of the current study are in agreement with what Ross et al. (2016) reported with regard to factors associated with dietary patterns, namely that duration of stay was not associated with the adoption of the host country's food culture among Liberian immigrants living in Ghana. In fact, these same authors observed that dietary patterns

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among Liberian immigrants remained different from those of the local population, irrespective of the number of years they had stayed in Ghana. The explanation for these contradictions could be attributed to the fact that these other studies (e.g. Deng et al., 2013; Lesser et al., 2014) were conducted on other continents, where circumstances are different.

In their study, Lesser et al. (2014) observed a different scenario from what our study and other studies have observed. Lesser et al. (2014) observed that a longer stay was associated with both negative and positive dietary changes. Positive changes included improved cooking methods, while negative dietary changes included increased consumption of red meat, fizzy drinks and desserts (Deng et al., 2013).

Limitations of the current study

The current study has a number of limitations. The use of non-probability sampling to select respondents for this study and the location where the study was conducted preclude any generalisation of the results. Therefore, the results reported here remain valid for the respondents involved and may be indicative of the problems experienced by sub-Saharan immigrants residing in South Africa. Consequently, validating these results in a different context and using a different sampling method would be valuable for further research. Another limitation of this study is that it did not measure food insecurity. Research has shown that there is high prevalence of food insecurity among immigrant groups, especially recent immigrants (Vahabi and Damba, 2013; Anderson et al., 2014). Thirdly, immigrants in this study were not grouped according to their immigration status. Research has shown that refugees and recent immigrants are highly vulnerable to food insecurity (Dharod et al., 2013). Lastly, the three-day food recall used in this study did not collect information on portion sizes, nor did it collect information on cooking methods and the ingredients used, especially for the traditional immigrant foods.

Conclusion and recommendations

To the best of our knowledge, the current study is the first of its kind to investigate dietary changes of immigrants in South Africa. An important finding of this study is that traditional food features very prominently in the dietary patterns of sub-Saharan immigrants. However, owing to high prices and unavailability of traditional ingredients, involuntary dietary acculturation takes place, forcing immigrants into bicultural eating patterns. High prices and unavailability of traditional ingredients could also make immigrants vulnerable to food insecurity. Therefore, further studies to identify similar-tasting, nutritious food items from the South African food culture – as substitutes for unavailable traditional ingredients – might be useful in preventing food rejection and informing culture-specific nutrition education programmes, which could contribute to positive nutritional transitions. Furthermore, our findings confirm that when dietary acculturation occurs, immigrant populations tend to adopt less healthy dietary patterns. In the long run, these unhealthy eating patterns could have negative implications, not only for the health of the immigrants themselves, but also for the health system in South Africa. This suggests the need to design targeted food security and culture-appropriate nutrition education programmes. Further studies to improve understanding of the severity and implications of unhealthy eating patterns among sub-Saharan immigrants in South Africa could help to inform policy and programmes aimed at improving the resettlement of immigrants.

Finally, the findings of this study suggest that there is no association between marital status, age, household income, duration of residence and the adoption of the South African food culture.

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SIMULATION OF POTENTIAL DISTRIBUTION AND MIGRATION OF *ALNUS SPP.* UNDER CLIMATE CHANGE

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Abstract. Plant migration is a well-known adaptation strategy of plant groups or species with evidence from historical to present observation and monitoring studies. Importance of N_2-fixing plants has increased in last decades. *Alnus* (alder) is an important plant group because of its nitrogen fixation ability. Alders are generally distributed in humid locations of boreal, temperate and tropical climate zones, where the nitrogen fixation is an important nitrogen source for other plants. To model the nitrogen fixation by alder, data about the global distribution of alder is absolutely required. In this study, a new method and model (Alnus-Distribution-Model (ADM)) are presented to predict the distribution of N_2-fixing genus on global scale and its migration in the future by using climate change scenarios up to 2300. Results of the study showed that the potential distribution of *Alnus spp.* not only depending on solitary use of climate variables, soil types and vegetation groups but on combined effect of all tree influencing variables. The ADM also presented that the *Alnus spp.* potentially will migrate mainly northwards in the northern hemisphere. This study covered basic approaches to understand the combine effect of climate, soil and vegetation on modelling of plant distribution and migration.
Keywords: *Alnus, plant distribution, plant migration, nitrogen fixation, climate change, RCP scenarios*

Introduction

Since the nitrogen is a key factor for carbon uptake processes by photosynthetic organisms, and the main resource of the available nitrogen for biogeochemical processes in ecosystems is the N_2 fixation by the symbiotic pathways between the host plants and N_2-fixing bacteria, the determination of distribution of the host plants has been gaining very important meaning and role in modelling of biogeochemical cycles in the ecosystems. Numerous biogeochemical and biome models use empirical or statistical methods to predict the nitrogen fixation by N_2 fixing plants (Prentice et al., 1992; Vitousek et al., 2002; Galloway et al., 2004; Esser et al., 2011). However, none of them considers the N_2 fixation by alders since there is limited information about the distribution of alder species on global scale. It makes difficult to implement the fixation process in biogeochemical, biome models to investigate the interactions between the carbon and nitrogen biogeochemical cycles. It is well known that plants can change their distribution with time, when environmental conditions (i.e. soil, climate etc.) and biological factors (i.e. plant–plant interaction) change in their distributed regions (Sauer, 1988; Dawis and Zabinski, 1992; Iverson and Prasad, 2002). Overpeck et al. (1991) and Bartlein et al. (1997) published new data about widening capability of trees due to the change in environmental conditions. Climate change in the 21st and 23rd centuries that mainly driven by emission change and its impacts on different sectors has been addressed by numerous studies and projects. It is also documented that rapid climate change may put some species at risk of extinction, and possibly reduce the functionality of ecosystems, which could have consequences for ecosystem processes such as global

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carbon storage and biodiversity (Thomas et al., 2003). Furthermore, a change in the land cover due to migration of plant species can also affect greenhouse gas concentration in the atmosphere, since for instance a migration of nitrogen fixing plants can influence the carbon uptake and nitrogen availability in soil (Kurz and Apps, 1999). In recent years, the importance of the nitrogen cycle for the sequestration of atmospheric carbon dioxide in the terrestrial biosphere has become obvious (Vitousek et al., 2002; Galloway et al., 2004; Reich et al., 2006; Wang et al., 2007; Esser et al., 2011). While the fixation of CO_2 by photosynthesis produces carbohydrates, nitrogen is required to bind carbon into phytomass. If the biospheric carbon pools increases, an adequate increase of the biospheric nitrogen pools is required. Atmospheric N_2 may be incorporated in the biosphere, but only a limited number of organisms are able to fix it, because of the high activation energy for the decomposition. These organisms are free-living or symbiotic cyanobacteria, actinomycetes, and bacteria in roots of host plants (Galloway, 2002). Not only N_2-fixing bacteria but also host plants that supply required energy for the fixation to the bacteria have enormous importance for the ecosystems. Most of the host plants belong to the families Fabaceae, Mimosaceae, Caesalpiniaceae (legumes) as well as to the *Betulaceae* (alder spp.), and they are called N_2-fixing plants (Saikia and Jain, 2007; Lepper and Fleschner, 1977). Because of their participation in the N_2 fixation, the modelling of distribution of the N_2-fixing plant species plays a key role in earth system and ecosystem modelling. A spatial change in distribution areas of the nitrogen fixers affects directly available nitrogen in soil, carbon uptake and allocations in the biosphere (Galloway et al., 2004). Numerous ecosystem and biogeochemical models aim to predict the nitrogen fixation by using empirical functions (Vitousek et al., 2002; Wang et al., 2007; Galloway, 2002; Esser, 2007). Still, the modelling of nitrogen fixation by alders is missing in most of the models. To predict the amount of fixed nitrogen under global climate change conditions, it is indispensable to have a mechanistic description of the N_2 fixation. And also, the description of the distribution of the symbiont's host plants, their density distribution in the vegetation types in which they occur, the type and the number of root nodules, and the activity of the nitrogen fixing enzyme systems in the nodules are needed. For instance, the density of alders in their native locations in Europe is mainly between 0 and 40% of total plant biomass (Skjøth et al., 2008). These percentage provide a possibility for a modelling the distribution and alders' biomass density according total plant biomass in a location by using models like Nitrogen–Carbon-Interaction-Model (NCIM) (Esser et al., 2011). Alder roots are generally infected with the symbiotic endophytic genus Frankia. As a symbiont, Frankia can convert atmospheric N_2 into reactive nitrogen usable by using the supplied carbohydrates from alders as energy source (Myrold and Huss-Dannel, 1994; Schwintzer and Tjepkema, 1990; Binkley, 1994). Thus, the N_2 fixation by alders can range from 20 kg·ha−1·yr−1 (Binkley, 1994) to 320 kg·ha−1·yr−1 (Van Miegroet et al., 1989). Therefore, alders play an important role in the respective ecosystems due to its ability to enrich poor soils with reactive nitrogen compounds. About 30 species belong to the genus alder, and to the family Betulaceae. The species are mainly distributed in the northern boreal and temperate zones e.g. *Alnus glutinosa* (L.) Gaerten, *A. incana* (L.) Moench, *A. viridis* (Chaix) D. C., *A. rubra* Bong., *A. oblongifolia Torr*, and *A. serrulata* (Ait.) Willd (Tutin et al., 2001). Some species extend into the subpolar zones, including A. hirsuta (Fischer) C.K. Schneider, *A. viridis* (Chaix) DC (Wiedmer and Senn-Irlet, 2006). In the Mediterranean zone occurs for example *A. cordata* (Loisel.) Duby. (Quézel et al., 1999). Numerous species are native to the mountains of the

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subtropical and tropical zones. *A. nitida* (Spach) Endl. occurs in the temperate Himalayas in altitudes from 1000 to 2900 m (Nasir, 1975). *A. nepalensis* D. Don is widely distributed in southeast Asia from subtropical China, Indochina, the Burmese (Shin) Hills, to the Himalayas in altitudes between 300 and 3000 m (Dai et al., 2004). Some alder species also distribute in the southern temperate zone, e.g. *A. acuminata* HBK, and the evergreen *A. jorullensis* Kunth are found in the Chilean Andes at high altitude (Reese, 2003). Within the distribution area of the alders the mean annual temperature is reported to range from −14 ºC to more than 20 ºC (NACS, 1980). The annual precipitation probably ranges from less than 150 mm (WRCC, 2009; Hagenstein and Rickets, 2001) to more than 5600 mm (Harrington, 1991). Alder species prefer poor soils of various particle sizes from gravel and sand to silt, loam, and even clay as well as organic soils. Most species occur on fenlands, in swamp areas, along brooks, rivers, and streams in bogs, but regularly not in riparian areas with highly varying water levels. However, some species such as *A. firma* Sieb. & Zucc. and *A. crispa* (Dryand. in Ait.) Pursh are distribute steep slopes. Several studies show that the main factors that influence the distribution of plant species in their natural ecosystem are climatic factors like temperature and precipitation (Woodward, 1996; Dukes and Mooney, 1999; Walther et al., 2002). Not only the climate change is a critical factor for plant distribution, but also the soil units via their different physical or/and chemical conditions can influence plant distribution (Brown, 1984; Min and Kim, 1999; Wu et al., 2011). Therefore, the soil units should be considered in the modelling studies about the prediction of plant distribution. Also, the occurrence of a plant species in its natural area is depending on plant–plant interactions. Plant species often favor to grow with specific other species (Pyke and Archer, 1991; Brooker, 2006). Several models like NCIM (Esser et al., 2011), LPJ-GUESS (Smith et al., 2001), and EMEP (Simpson et al., 2012) consider plant–plant interactions due to use potential natural vegetation groups or biome units in the model simulations.

In this paper, the aims were reported for modelling the global distribution of the N_2 fixing host genus alder, and then the effect of climate change effect on the globally *Alnus* distribution. To predict the global distribution of alders, an available gridded data sets on climate, soil units and potential natural vegetation groups will be used. The individual contribution of each data type was tested for the correctness of the predicted distribution. This work should be a first step to predict the potential occurrence and distribution of alders depending on their climate requirements, soil conditions, and plant–plant interactions. This study should also give the basic information for implementation of N_2-fixation by alders in biogeochemical and ecosystem models since N_2 fixation is directly depending on the occurrence of N_2-fixing plants in the terrestrial ecosystem.

Materials and methods

In this paper, a new model based on four progress steps was developed for the predicting of the potential distribution of alder spp. on the global scale. This new model is called “Alnus-Distribution-Model (ADM)”. In the first step, the values of annual average temperature and precipitation were used from Leemans and Cramer 0.5º degree grid element global climate database (Cramer and Leemans, 1991) to define a bioclimatic niche of *Alnus spp*. The climate database includes 30 years (1961–1990) average of the climate parameters for 0.5º resolution on the global scale. In the second

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step, the climate based ADM was extended with soil units by using the FAO soil classification (1974) in "Soil Types of the World" (FAO-Unesco, 1974). In the third step, the climate parameters based ADM was extended with potential natural vegetation groups after Esser et al. (2011). The vegetation data set is our own digitized database from the "Atlas for Biogeography" after Schmithüşsen (1976). The vegetation map after Schmithüsen comprises 176 vegetation units globally. These 176 vegetation units were aggregated in 31 potential natural vegetation groups in the research group at the institute, and it published in the study Esser et al. (2011). In the fourth step, all three methods were merged to predict the potential alders' distribution by the ADM. The used climate, soil, and vegetation data sets are on identical global grid resolution, i.e. half degree longitude and latitude as commonly used by global vegetation models. 62483 grid elements are characterized for the land areas excluding Antarctica. Each grid element is characterized by its lower left (south–east) corner coordinate in decimal degrees.

Distribution data for alders

For the construction of ADM, the global distribution data for of the *Alnus spp*. were extracted from seven databases (Tropicos.org, 2009; eFloras, 2008; WWF, 2009; Tutin et al., 2001; US Forest Service, 2008; USDA-NRCS, 2009; Li and Skvortsov, 1999). The number of data for alder occurrence is very unevenly distributed worldwide. The name of the alders' species, the altitude, and the coordinates of the origin place were collected. A total of 308 locations including the data were extracted. All species of genus *Alnus* Mill. of *Table 1* are represented in the 308 locations. The lifespan of alders ranges between 40-100 years (Harrington et al., 1994; Claessens et al., 2010). It is assumed that a change in the 30-year annual average of climate conditions (i.e. temperature and precipitation) can change the suitable climate conditions in the distribution area and force the migration of alder species. Unfortunately, there is data for validation of this assumption in the academic literature databases. Therefore the 30-year period was used for the prediction of alder distribution and migration. This is a weak point of the model, and may be changed in the future by long-term observation studies.

Table 1. *The global distributed 34 alder species, which were used for selection of the locations and the relevant climate, soil, and vegetation parameters. The species names are according to the publication from Chen and Li (2004).*

Species name	Species name
A. acuminata **HBK**	*A. matsumurae* Callier
A. barbata **C. A. Mey**	*A. maximowiczii* Callier
A. cordata **(Loisel.) Duby.**	*A. nepalensis* D. Don
A. cremastogyne **Burkill**	*A. nitida* (Spach) Endl.
A. crispa **(Dryand. in Ait.) Pursh**	*A. oblongifolia* Torr
A. fernandi-coburgii **C.K. Schneider**	*A. orientalis* Decne
A. firma **Sieb. and Zucc.**	*A. pendula* Matsum
A. formasana **(Burkill) Makino**	*A. rhombifolia* Nutt.
A. fruticosa **(Du Roi) Spreng.**	*A. rubra* Bong.
A. glutinosa **(L.) Gaerten**	*A. rugosa* (Du Roi) Spreng.
A. hirsuta **(Fischer) C.K. Schneider**	*A. serrulata* (Ait.) Willd
A. incana **(L.) Moench**	*A. sieboldiana* Matsum
A. inokumae **S. Murai and Kusaka.**	*A. sinuata* (Regel) Rydb.

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***A. japonica* (Thunb.) Steud.**	*A. subcordata* C.A. Mey
***A. jorullensis* Kunth**	*A. tenuifolia* Nutt.
***A. mandshurica* C. K. Schneider**	*A. trabeculosa* Hand. and Mazz
***A. maritima* (Marsh.) Nutt.**	*A. viridis* (Chaix) D. C.

"Clim"

I determined the grid elements, in which alders occur in the 308 sites. All further analyses were made by using the gridded data sets. First, the mean annual temperature (T_{ann}) and annual total amounts of precipitation (P_{ann}) were extracted from the gridded climate data set for the sites of alders. The altitudes of the alder locations within a grid element may deviate from the mean altitude of the grid element. Therefore, corrections of the gridded climate data were sometimes necessary. For this purpose, the altitude of the site that was given in the original databases was used. If altitudes were lacking, it was determined from the GTOPO30 global elevation dataset (GTOPO30, 2010). If the altitude could not be determined, the side was eliminated. The nearby climate stations were selected from Walter and Lieth (1961–1967), Müller (1982) and Mitchell and Jones (2005). The arrays of T_{ann} and P_{ann} were plotted for the 308 alder sites. Three linear functions were then determined which envelop the field of climate data of the alder sites. The T_{ann} and P_{ann} values of the 308 locations were presented in the *Fig. 2*. three linear functions F1–F3 were fitted to the six cardinal points P1–P6:

F1(P1, P2); F2(P3, P4); F3(P5, P6). The three linear functions that form the borderlines of alder distribution in the temperature–precipitation matrix are:

$$F_1(x) = -2.04 * x + 172.58 \quad \text{(Eq. 1)}$$

$$F_2(x) = -561.58 * x + 16141.87 \quad \text{(Eq. 2)}$$

$$F_3(x) = 110.67 * x + 1658.64 \quad \text{(Eq. 3)}$$

The *x* equals to T_{ann} (ºC) and $F_{(1,2,3)}(x)$ to P_{ann} (mm). In *Figure 2* plot of these functions can also be found.

To determine the potential distribution areas for alders, the following climate based method was used:

$$D_{Clim,i} = \begin{cases} true, if\ Clim_{T_{ann},P_{ann},i}\ inside\ climate\ matrix\ field \\ false, else \end{cases} \quad \text{(Eq. 4)}$$

where *i* is grid number of half degree grid element, T_{ann} (ºC) is mean annual temperature, and P_{ann} (mm) is annual total amounts of precipitation of the grid element, respectively. The distribution of alder species based on climate parameter ($D_{Clim,i}$) is true in a grid element if the certain criteria of the grid element are fulfilled (see *Eq. 4*).

"Soil"

For this aim, the FAO soil units from the "Soil Types of the World" database, which

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includes 129 soil units for the 0.5º grid cells of the terrestrial biosphere (excl. Antarctic) (FAO-Unesco, 1974) were used to enhance the climate based ADM for prediction of potential alders distribution. The soil units of the 308 study sites, in which the alders natively occur were recorded as suitable soil types for the alder distribution. Thereby, the soil units were used as additional determinants for the alder occurrence. If a soil unit were present in only one grid element, it was not considered in the modelling study. Grid elements were marked as potential alder habitats, if they were within the climate field limited by the three linear functions of the temperature–precipitation field, and have suitable soil unit, which occurs in more than one grid elements with alder distribution. For this step, the following equation was used:

$$D_{Soil,i} = \begin{cases} true, if \begin{cases} Soil_i = Soil_a \\ D_{Clim,i} = true \end{cases} \\ false, else \end{cases} \quad \text{(Eq. 5)}$$

where i is grid number of half degree grid element, $Soil_i$ is the soil unit of the grid element, and $Soil_a$ is the soil unit of the grid elements with data record about alder distribution in 308 study sites, respectively.

"Veg"

In this step, the 31 potential natural vegetation groups according to the study from Esser et al. (2011) were used to investigate the correlation between the alder distribution and climate–vegetation aspect in this study. The potential natural vegetation groups, in which alders occur natively were marked as suitable vegetation groups for alder distribution. Thereby, these potential natural vegetation groups were used as additional determinants for alder distribution. If a vegetation group was recorded in only one grid element, it was not considered in the modelling study. Grid elements were marked as potential alder habitats if they were within the climate field limited by the three linear functions of the temperature–precipitation field, and have suitable potential natural vegetation group which occurs in more than one grid elements with alder distribution. For this step, the following equation was used:

$$D_{Veg,i} = \begin{cases} true, if \begin{cases} Veg_i = Veg_a \\ D_{Clim,i} = true \end{cases} \\ false, else \end{cases} \quad \text{(Eq. 6)}$$

where i is grid number of half degree grid element, Veg_i is the vegetation type of the grid element, and Veg_a is the vegetation type of the grid elements with data record about alder distribution in 308 study sites, respectively.

"All"

In this step, all three method were combined for modelling of potential alder distribution. The verified the soil units, and the potential natural vegetation groups which occur in the grid elements with alder sites were used together as additional determinants for alder occurrence. The equation of this step is as follows:

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$$D_{All,i} = \begin{cases} true, if \begin{cases} D_{Clim,i} = true \\ D_{Soil,i} = true \\ D_{Veg,i} = true \end{cases} \\ false, else \end{cases} \quad \text{(Eq. 7)}$$

where *i* is grid number of half degree grid element.

Migration of alder species in 2100 and 2300

To predict the migration of alder species up to 2300, data for T_{ann} and P_{ann} were needed. For this step, the mean annual value of temperature and precipitation of four RCP (Representative Concentration Pathway) scenarios (i.e. RCP 2.6, RCP 4.5, RCP 6.0 and RCP 8.5) were selected from the CCSM4 data model outputs for the period between 2000 and 2300. The climate data of the four different RCP scenarios was used for checking the potential effect of variability in temperature and precipitation on plant distribution (i.e. *Alnus spp*.) due to change in anthropogenic emission of greenhouse gases, technology and population density according to the RCP scenarios. Since the used climate data for the prediction of alder distribution in the “Clim” stage were the 30-year annual average data from Leemans and Cramer data base, 30-year annual average value of T_{ann} and P_{ann} from 2071 to 2100, and from 2271 to 2300 were used for prediction of alder distribution in 2100 and 2300, respectively. The 30-year periods give plants the possibility for adaptation to the climate change in a location. To avoid the jump of alder species over long distance (i.e. more than one grid cell), it is also assumed that the alder cannot migrate to a grid cell if at least one of the neighbor grid cell was not marked as a potential distribution grid cell.

Observations and statistical analyses

The Global Biodiversity Occurrence Data Base (GBIF) (GBIF, 2010) was used for the evaluation of the model results. The database includes 237178 data records about the alder occurrence worldwide. Majority of these observations crowds together in a few regions of the world, while data in other regions are very scarce, so that the global coverage is very uneven. The database includes a global distribution map as well as the opportunity to download information amongst others the coordinate, name of the occurred alder species, and basis of records (unknown, herbarium, observed or specimen) in the locations. In the *Table 4*, 49 countries were presented, which were extracted from the database with data records about the alder distribution. Countries with just one data record for alder distribution or data records without the coordinate of the location or with the “unknown” basis of records were not considered in this study. Therefore, 215444 of 237178 were selected as useful data records in the 49 countries (see *Table 4*).

The prediction of the ADM model was validated with the data from the GBIF database for each step as well as for analyzing correlations between the observed and predicted data by calculation regression coefficient, index of agreement d (Willmott, 1982) (see *Eq.* 9), mean absolute error (*MAE*) (see *Eq. 8*) to determine the best method for the prediction of the alder distribution. The used *MAE* and d equations are:

$$MAE = \frac{1}{n}\sum_{i=1}^{n} |P_i - O_i| \quad \text{(Eq. 8)}$$

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$$d = 1 - \frac{\sum_{i=1}^{n}(P_i-O_i)^2}{\sum_{i=1}^{n}((|P_i-\bar{O}_i|)+(|O_i-\bar{O}|))} \quad \text{(Eq. 9)}$$

where P is the number of the simulated grid cells with potential alder distribution in related locations and O is the number of the observed grid cells with alder distribution, i a sample, n the number of samples, overbar represents mean values, and d is the index of agreement, respectively.

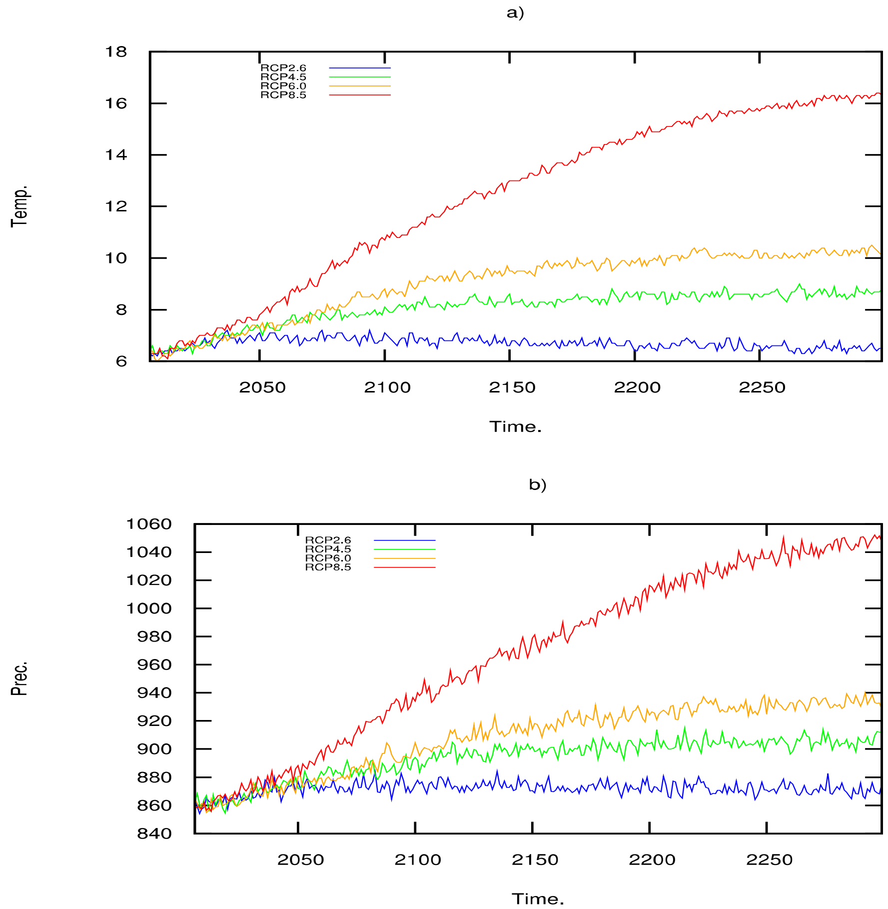

Figure 1. *The change in global average temperature and precipitation (2006–2300) of CCSM4 model which driven by four RCP emission scenarios.*

Results

Evaluation of distribution methods

In *Figure 2*, the distribution of the 308 data points in the field of T_{ann} and P_{ann} is shown. In the distribution regions, there is a lower limit of annual precipitation, which excludes the occurrence of alders. This lower limit depends also on T_{ann}. At the alder distribution sides, when the T_{ann} around −10 ºC or colder, P_{ann} limit is about at 190 mm. When T_{ann} is around 28ºC, the alders need about 115 mm annual precipitation for their existence. Since alders occur at low precipitation values mainly along rivers and brooks, it is assumed that the occurrence of alders in areas with low P_{ann} is due to the probability of the suitable soil water content.

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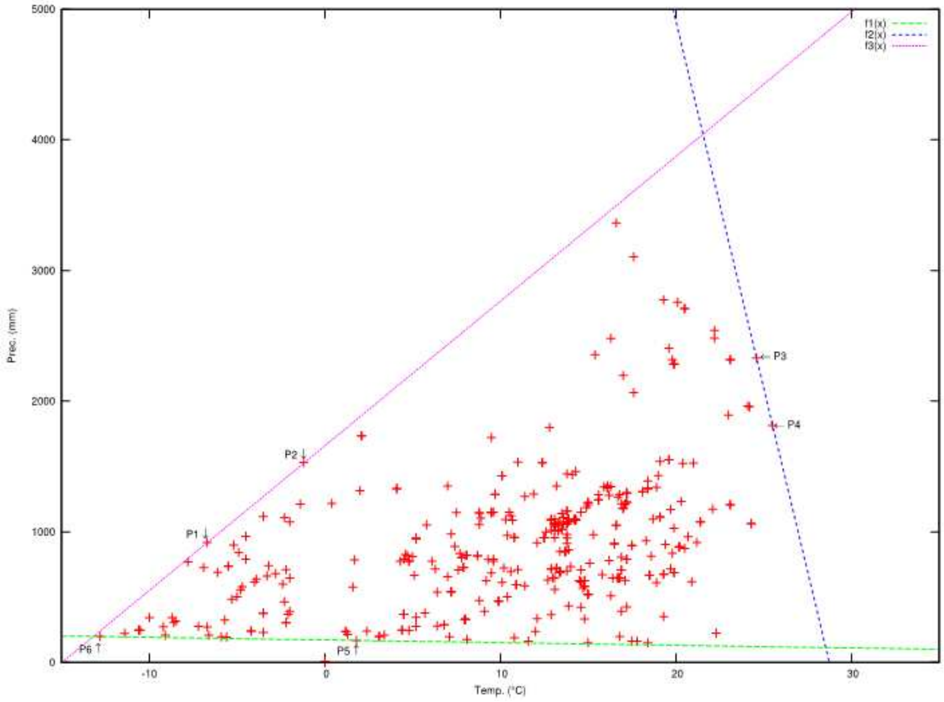

Figure 2. *The temperature–precipitation field of the 308 data points which were extracted from seven data bases (Tropicos.org, 2009; eFloras, 2008; WWF, 2009; Tutin et al., 2001; US Forest Service, 2008; USDA-NRCS, 2009; Li and Skvortsov, 1999) as sites of alder occurrence. The cardinal points P1,...,P6 define the borderline of the distribution of alder in this field. They define the three linear functions 1 through 3 which were used to select appropriate grid elements from a global 0.5° grid of climate data (Cramer and Leemans, 1991).*

From the selected six cardinal points P1...P6 to define the borderline of the alder distribution in a matrix (see *Figure 2*), the point P1, P2 and P6 refers to *Alnus viridis*, which occurs in the northern boreal regions of Asia, Europe, and North America. In temperate regions, *A. viridis* may occur at high elevations (Kamruzzahan, 2003). P_{ann} range for *A. viridis*. is between 150 and 3000 mm yr^{-1} in its native distribution areas (Racine et al., 2001). The points P3 and P4 belong to the two species *Alnus acuminata* the Andean alder, and *Alnus jorullensis* the Mexican alder, which are native to the mountains of Central and South America. Their distribution defines the upper temperature limit of the alder distribution, which seems to be below 30 ºC average annual temperature. In the regions, P_{ann} may range from 500 to 4000 mm yr^{-1}. The point P5 refers to *Alnus rhombifolia*, which occurs in the lower areas of the northern Pacific coast of North America from humid to per-humid climates (USDA-NRCS, 2009). The P_{ann} within the distribution areas of *A. rhombifolia* varies from 508 to 3175 mm per year, and the lowest temperature is −4.4ºC (USDA-NRCS, 2009).

Further stages of the model

The climate based ADM was refined by means of the soil units. The soil units that recorded in the 308 grid elements are shown in *Table 2*. The considered alder distribution areas involve 53 of 130 FAO soil units. Lithosols and Cambisols are the dominated soil units in the 308 distribution areas. About half the grid elements include the two soil units. Although, most of the alder species prefer to distribute in wet soils

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and in soils with high water availability, the Gleysols were found only in 21 of 308 grid elements. Gleysols are wetland soils and categorized in FAO-UNESCO soil database as a hydromorphic soil group, which are influenced by groundwater for a long period to develop a characteristics gleyic pattern, and are mainly covered by swamp vegetation (FAO-Unesco, 1974). The soil units, which were present in only one grid element, were not considered in this work.

Table 2. *Soil units related to Soil Map of the World of FAO-UNESCO (1974) which dominate in the 0.5° grid elements where alder species occur according to the GBIF database. The bold lines are the main groups of the related sub group for soil types according to FAO-UNESCO Soil Map of the World.*

Nr. of the grids	**FAO unit**	**Soil name**
78	B	Cambisols
21	BK	Calcic Cambisol
19	BD	Dystric Cambisol
19	BE	Eutric Cambisol
15	BH	Humic Cambisol
2	BG	Gleyic Cambisol
2	BX	Gelic Cambisol
75	I	Lithosols
35	A	Acrisols
26	AO	Orthic Acrisol
4	AF	Ferric Acrisol
4	AH	Humic Acrisol
1	AG	Gleyic Acrisol
1	AP	Plinthic Acrisol
22	P	Podzols
17	PO	Orthic Podzol
5	PL	Leptic Podzol
20	G	Gleysols
15	GD	Dystric Gleysol
4	GE	Eutric Gleysol
1	GM	Mollic Gleysol
18	L	Luvisols
7	LC	Chromic Luvisol
6	LO	Orthic Luvisol
5	LA	Albic Luvisol
14	T	Andosols
7	TV	Vitric Andosol
5	TH	Humic Andosol
2	TM	Mollic Andosol
13	H	Phaeozems
6	HG	Gleyic Phaeozem
5	HL	Luvic Phaeozem
2	HH	Haplic Phaeozem
7	R	Regosols
4	RX	Gelic Regesol
2	RC	Calcaric Regesol
1	RD	Dystric Regesol
5	Y	Yermosols
3	YL	Luvic Yermosol

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2	YK	Calcic Yermosol
4	N	Nitosols
4	NE	Eutric Nitosol
4	O	Histosols
4	OX	Gelic Histosol
3	J	Fluvisols
3	JE	Eutric Fluvisol
3	U	Rankers
3	X	Xerosols
2	XH	Haplic Xerosol
1	XL	Luvic Xerosol
1	F	Ferrasols
1	FX	Xanthic Ferrasol
1	K	Kastanozems
1	KL	Luvic Kastanozem
1	W	Planosols
1	WE	Eutric Planosol
1	ICE	Ice

In *Table 3*, the potential natural vegetation groups of the 308 grid elements after Esser et al. (2011) and their vegetation units which occur in digitized version of the atlas for bio-geography after Schmithüsen (1976) were shown. 50 of 176 vegetation units according to Schmithüsen (1976) were recorded in the 308 grid elements. The most common vegetation units in the distribution areas was the potential natural vegetation group "Temperate deciduous forests" (68 of 308 grid elements). In 97 locations, the tropical and subtropical potential natural vegetation groups were recorded. In 32 locations, dry vegetation units ("Open conif. dry woodland", "Conif. dry forest", "Puna dry steppe", "Drought-deciduous and part evergreen thorn bush formation", "Artemisia dry steppe", and "Trop. lowland dry forest") were found. In those locations, the alders may distribute in moist areas along rivers and streams. For instance, the "drought-deciduous and part evergreen thorn bush formation" (7 sites) occurs on the east slopes of the Argentinian and southern Bolivian Andes, where a number of brooks and rivers are present. There are also 33 grid elements with sclerophyllous formations of Mediterranean type climates. These sites may also be supported by water currents occurring in these formations.

Table 3. *The potential natural vegetation groups according to the study from Esser et al. (2011) (name with bold character) and the vegetation units according to Schmithüsen (1976) which occur in the 0.5° grid elements which include the data records about alder occurrence. The left column gives the respective number of grid elements.*

Nr. of records	Name of the vegetation group
68	Temperate deciduous forests
22	Cold-deciduous broadleafed forest w. evergreen conif. trees
20	Cold-deciduous mesophytic broadleafed forest
10	Submediterranean cold-deciduous broadleafed forest
6	Mountain cold-deciduous mesophytic broadleafed forest
4	Cold-deciduous mesophytic broadleafed forest w. Quercus
3	Cold-deciduous broadleafed forest w. evergreen broadleafed trees
3	Mountain cold-deciduous broadleafed forest w. conif. trees

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50	Tropical mountain forests
24	Tropical evergreen cloud forest
12	Tropical deciduous moist mountain forest
9	Tropical mountain rain forest
5	Tropical evergreen oak-pine forest
43	Boreal evergreen conif. forest
21	Boreal evergreen mountain conif. forest
15	Boreal evergreen conif. forest w. cold-deciduous broad-leafed
7	Boreal evergreen conif. forest
33	Mediterranean sclerophyll formations
16	Sclerophyllous forest w. Quercus ilex
15	Sclerophyllous forest w. Olea
2	Sclerophyllous forest w. Quercus suber
32	Boreal woodlands
32	Boreal, subpolar open conif. woodland
22	Temperate woodlands
16	Open conif. dry woodland
4	Cold-deciduous tree steppe
2	Conif. dry forest
10	Subtropical evergreen forests
6	Laurel mountain forest
2	Laurel forest w. conif. trees
1	Subtropical semi-deciduous rain forest
1	Laurel forest
10	Temperate evergreen forests
7	Temperate conif. rain forest
2	Extra-boreal mountain conif. forest
1	Extra-boreal mountain conif. forest w. Pinus
9	Tropical Paramo woodlands
7	Paramo heath
2	Paramo laurel woodland
8	Puna steppes
6	Moist Puna steppe
2	Puna dry steppe
7	Mediterranean woodlands, shrub formations
5	Drought-deciduous, part evergreen thorn bush formation
1	Open sclerophyllous woodland
1	Sclerophyllous garrigue
7	Xerophyte formations
7	Tropical-subtropical deciduous scrub
5	Temperate shrub formations
2	Artemisia dry steppe
2	Hard, thorn pillow mountain formation
1	Peat-moss raised bog w. conif. trees
5	Tropical lowlands dry forests
5	Tropical deciduous dry forest
4	Tropical lowlands rain forests
2	Tropical evergreen lowland rain forest
1	Tropical semi-deciduous lowland rain forest
1	Tropical deciduous moist forest
2	Subtropical savannas
1	Sclerophyllous shrub formation

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1	Thorn savanna
2	Temperate steppes, grasslands
2	Transitional steppe
1	Subtropical deciduous forests
1	Subtropical cold-deciduous conif. swamp-forest
1	Subtropical halophyte formations
1	Saltings or coastal dune vegetation
1	Tropical savannas
1	Open evergreen savanna woodland
1	Tropical Paramo grasslands
1	Paramo grassland
1	Ice

Observed alder distribution

In *Table 4*, data about the 49 countries with data records for alder distribution in GBIF database were presented. The 49 countries include 215444 data records with coordinates of the locations, name of the occurred alder species, and the basis of the records for alder occurrences. The countries were ordered after having most data records (i.e. countries with most data records first). The first 20 countries in the *Table 4* included the most data records for alder distribution in the GBIF database (see *Table 4* column "Rec."). The total number of records in these countries is 208181 of 215444 in 1866 of 4098 half degree grid elements (see *Table 4* columns "Rec.", and GBIF 05"). Also, the first 20 countries (14 in Europe, 3 in South America, 2 in Asia, and 1 in Central America,) in the *Table 4* include 97% of the useful data records and the most data density for alder distribution. Each country has over 100 data records, and also 5 data records per half degree GBIF grid cell. Thus, these 20 countries were used for the validation of model prediction about alder distribution. The other 29 countries in the *Table 4* included the rest of data records for the alder distribution in GBIF database. Two countries after the middle line the table (US and Canada) have indeed high data records but less data density (records number per grid elements). Therefore, these countries were not considered within the 20 countries. Countries with only one data record in GBIF data base were also not considered and not presented in this paper.

Table 4. *Analysis of the data distribution in the GBIF database (GBIF, 2010) which was used for the validation of the ADM model results for 49 countries with data records for alder distribution.*

Contry	Nr. of records	Nr. of 0.5 grids	Nr. of GBIF grid
UK	44 911	146	146
NL	35 785	17	17
BE	23 668	17	9
SE	22 889	321	292
FI	19 292	253	246
FR	15 868	261	160
NO	15 761	271	237
DE	11 905	191	191
IE	7521	43	43
ES	3819	212	134
JP	1993	163	101

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PL	1898	168	46
MX	1362	715	132
TW	480	14	13
AT	393	36	28
PT	169	48	33
KR	132	40	9
AR	117	1138	23
BO	112	365	10
EC	106	83	19
US	5166	4469	1413
CA	1439	7004	555
RU	143	14 283	73
CN	80	3834	3
PE	50	427	30
CO	46	377	16
PA	39	30	2
DK	38	30	6
IT	34	145	14
CH	30	19	10
GT	27	37	10
GR	23	61	8
CZ	23	63	15
PK	19	326	4
NZ	17	135	10
ZA	14	479	2
NP	10	53	4
TR	8	332	4
IN	8	633	5
HN	8	42	3
AU	8	2826	3
RO	7	111	4
VN	5	105	2
CL	5	351	3
VE	3	304	2
BG	3	49	2
IL	2	6	2
HU	2	45	2
GL	2	2770	2

Meaning of the columns: (Country) name of the countries; (Rec.) Number of data records for alder distribution in each country (countries with just one data record are not shown); (Grid 0.5) Number of half degree grid elements of the country; (GBIF 0.5) Number of half degree grid elements with data records about the alder distribution. The first 20 countries have in the GBIF grid cells minimum 100 data records and five data records per grid.

Validation of the methods

Since the 20 countries had the most density for data records about the alder distribution in GBIF database, a statistical analysis between the observed and predicted alder distribution was done in these countries to find out, which method of the four methods (“Clim”, “Soil”, “Veg”, and “All”) is more suitable for the modelling of alder distribution. In the *Figure 3* the results of the correlation and statistical analyses

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between the observed and predicted number of half degree grid elements with data records about alder distribution were presented. The correlation functions (f (x)), 1:1 lines, correlation coefficients (r^2), index of agreement (d), and mean absolute error (*MAE*) were also presented in the scatter plots. The actual data of the scatter plots may be found in the *Table 5* columns "Grid", "Soil" "Veg", and "All" respectively. The r^2 values of the correlation analyses ranged between 6 and 84%. The lowest correlation was found between the observed and "Clim" method based ADM results with r^2 = 6%. The d and *MAE* values of this correlation analysis were 0.36 and 117, respectively (see *Figure 3a*). The "Clim" method shown also a large intercept with 117 grid elements. The "Soil" method shown similar correlation with the observed data as the "Clim" method (see *Figure 3b*). The correlation coefficients r^2 between this method and observed data were 11%. The value of d for this method was 0.5, where the *MAE* value was 86. The method "Veg" provided a better correlation with the observed data (see *Figure 3c*). The values of index of agreement and mean absolute error shown quite good results with d = 0.93, and *MAE* = 28, respectively. But the best correlation coefficient (r^2= 84%) were found between the observed and "All" method based ADM results (see *Figure 3d*). And also the highest d value with 0.96, and the lowest *MAE* value with 27 were found between the "All" method based ADM and observed data. The intercept of this method was around 27 grid elements. Thus, the correlation analyses shown the best performance between the "All" method based ADM results and the observed data in the high relevant 20 countries. Because of the best r^2, d, *intercept*, and *MAE* values, the "All" method based ADM was used to predict the potential alder distribution areas globally.

The figures in the *Appendix* give an overview about the statistical analyses between the results of ADM by using each single parameter and observation in the 20 countries, and about the results of "CLIM", "Soil" and "Veg" methods on global scale.

Table 5. *Analysis of the data distribution in the GBIF database (GBIF, 2010) which was used for the validation of the ADM model results for 49 countries with data records for alder distribution, and comparison with model results using different constraints besides climate.*

Country	Tot. 0.5 Grid	Clim	Soil	Veg	All
UK	146	146	138	118	110
NL	17	17	17	16	16
BE	9	17	17	17	17
SE	292	319	319	302	302
FI	246	247	247	247	247
FR	160	261	238	256	233
NO	237	267	267	172	172
DE	191	191	165	184	162
IE	43	43	43	38	38
ES	134	212	206	181	177
JP	101	163	163	163	163
PL	46	168	134	168	134
MX	132	652	329	122	99
TW	14	14	14	13	13
AT	28	36	23	34	23

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PT	33	48	48	47	47
KR	9	40	40	40	40
AR	23	950	746	34	32
BO	18	259	238	24	24
EC	19	60	59	18	18
US	1413	3925	2920	2130	1912
CA	555	4625	3139	3403	2168
RU	73	10 695	7952	7018	4881
CN	3	3070	2980	1411	1379
PE	30	240	233	53	53
CO	16	110	105	51	51
PA	2	5	5	4	4
DK	6	30	30	30	30
IT	14	143	134	137	129
CH	10	19	16	14	11
GT	10	27	18	14	11
GR	8	61	61	58	58
CZ	15	63	59	63	59
PK	4	279	237	25	24
NZ	10	135	135	7	7
ZA	6	461	308	5	5
NP	4	43	43	3	3
TR	4	332	325	211	204
IN	5	1204	957	187	171
HN	3	30	26	18	16
AU	3	2739	1773	169	157
RO	4	111	94	104	93
VN	2	75	75	29	29
CL	3	217	206	95	95
VE	2	192	183	58	58
BG	1	49	34	48	34
IL	1	6	6	0	0
HU	2	45	29	45	29
GL	3	388	131	0	0

Meaning of the columns: (Country) name of the countries; (Tot. 0.5 Grid) Number of half degree grid elements with data records about the alder distribution; (Clim) Number of simulated half degree grid elements with potential alder distribution by using “Clim” method based ADM; (Soil) by using “Soil” method based ADM; (Veg) by using “Veg” method based ADM; (All) by using “All” method based ADM.

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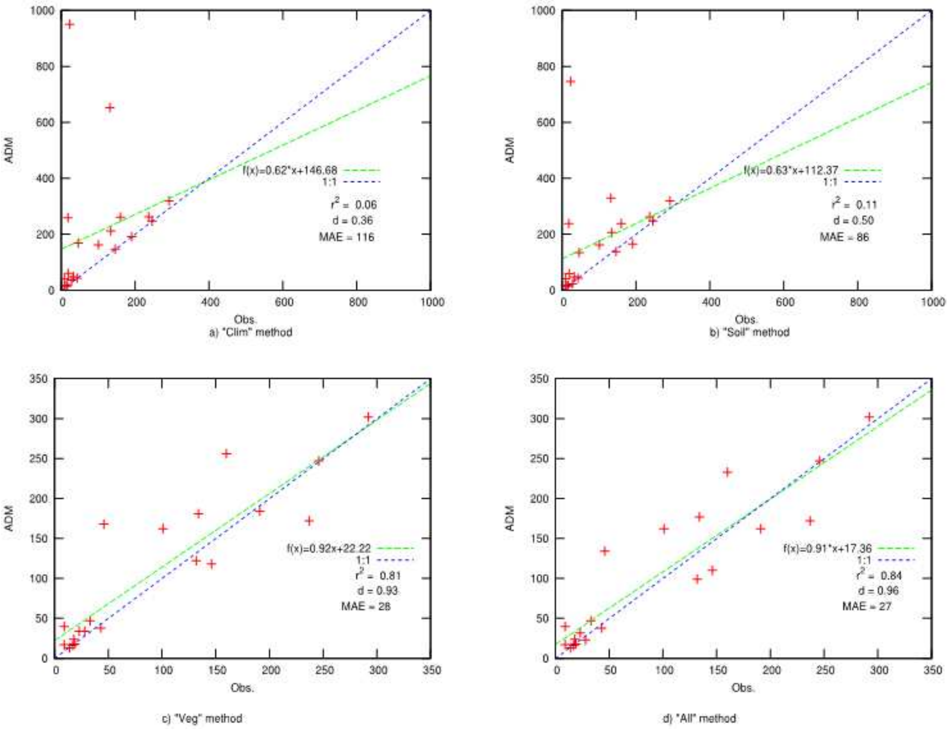

***Figure 3.** The correlation between the observed and predicted alder distribution in half degree grid elements in 20 countries. Countries from the Table 4 with minimum 100 data records and five data records per each noted half degree grid cell were considered. The regression and 1 : 1 lines are shown along with correlation coefficient (r), index of agreement (d), mean absolute error (MAE).*

Global potential alder distribution

The predicted alder distribution by using "All" method based ADM was shown in *Figure 5*. It is to see that alder has a large potential distribution areas in Asia and North America. In comparison to the global alder distribution on GBIF map (see *Figure 4*) the ADM also predicted the potential distribution in several grid elements of South America, Africa, and Australia (see *Figure 5*). The ADM predicted the alder distribution in 1898 grid elements in the 20 countries where the GBIF database has records in 2066 grid elements (see *Table 5* columns "Grid" and "All"). Most of the eliminated grid elements have suitable soil units but not vegetation types for the potential alder distribution in these countries. For example, eliminated grid elements in Norway have the soil unit "Lithosols" and the vegetation type "Mountain vegetation above the tree line". "Lithosols" are the second largest occurred soil units in the 308 grid elements (75 of 308) of the evaluation's grid elements (see *Table 2*). These grid elements have the suitable climate conditions and soil units but not the vegetation types. Most eliminated grid elements in the 20 countries after using "Soil" and "Veg" methods in ADM were found in Mexico. The dominant soil units in Mexico are "Leptosols", "Regrosols", and "Calcisols" (FAO-Unesco, 1974). Only "Regrosols" were presented in 308 evaluation's grid elements (see *Table 2*). Also, the dominated vegetation types are "Shrub desert", "Thorn savanna", and the "Open deciduous small leafed" in Mexico. Only the vegetation units "Thorn savanna" were recorded in one of 308 grid elements. In Russia and China, the ADM has shown the potential alder distribution in 67, and 460

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times more grid elements than the GBIF database records. In Russia, grid elements with potential alder distribution have the suitable climate conditions, soil units ("Lithosols" and "Cambisols") as well as the potential natural vegetation groups ("Boreal coniferous forest" and "Boreal woodlands"). These two soil units were recorded in 45 of the 308 evaluation's grid elements (see *Table 2*) and the vegetation types in 75 of the 308 grid elements (see *Table 3*). In China, same vegetation types are also the dominant vegetation types in potential distribution areas. However, the mostly coming soil units in those areas are the "Cambisols", "Gleysols" and "Acrisols". The three soil units were found in 121 of 308 grid elements. In comparison to the 20 countries, the US and Canada have also large data records but appreciably low data density per grid elements. The ADM predicted the alder distribution in 499 grid elements more in US and in 1613 in Canada than the GBIF database. In the regions, "Lithosols", "Podzols", "Luvisols", and "Phaozems" are mostly recorded soil units (FAO-Unesco, 1974). The potential natural vegetation groups are mainly "Temperate deciduous forests", and "Mediterranean sclerophyll formations" in the US, "Boreal evergreen conif. forests" in Canada.

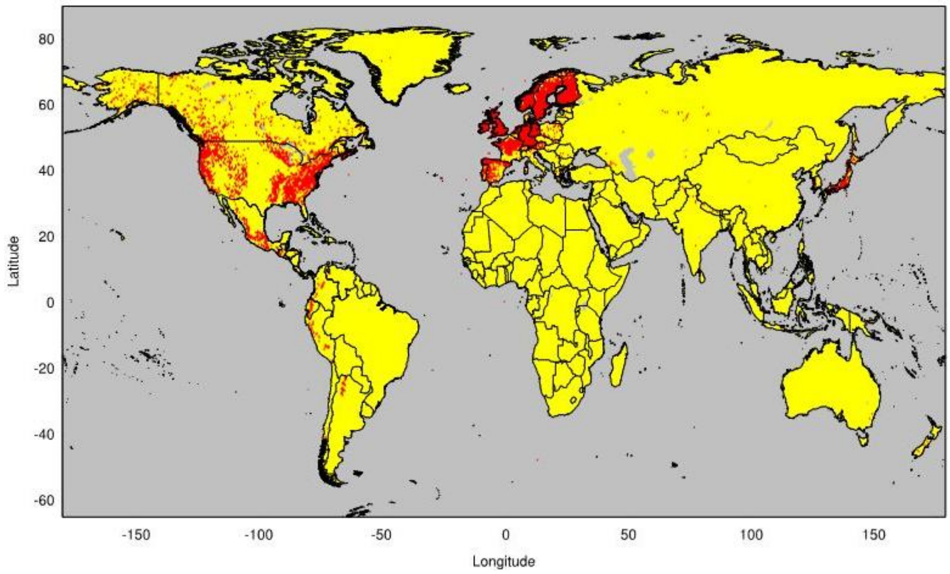

Figure 4. *Distribution of alders (red) according to the GBIF database (GBIF, 2010). For the locations with yellow colour there is no data record in the database.*

It is generally to see that the "Veg" method eliminated more grid elements than the "Soil" method in 12 of the 20 countries as well as in Russia, in China, and in the US. In France, Germany, Poland, Austria, and Canada more grid elements were eliminated by using the "Soil" method than the "Veg" method.

The results shown that the existence of alders in natural ecosystems is not only depending on climate conditions but also on soil types, and vegetation units. The potential distribution of alders mainly occurs in Northern Hemisphere, but also occurs in quite few locations in south hemisphere with adequate climate conditions, soil types and vegetation units (see *Figure 5*). Since the alders can fix atmospheric nitrogen, consideration of the nitrogen fixation by alders in their distributional areas in ecosystem and biogeochemical models gives the opportunity to investigate and predict the nitrogen

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fixation impacts on CO_2 uptake, and carbon storage in the.

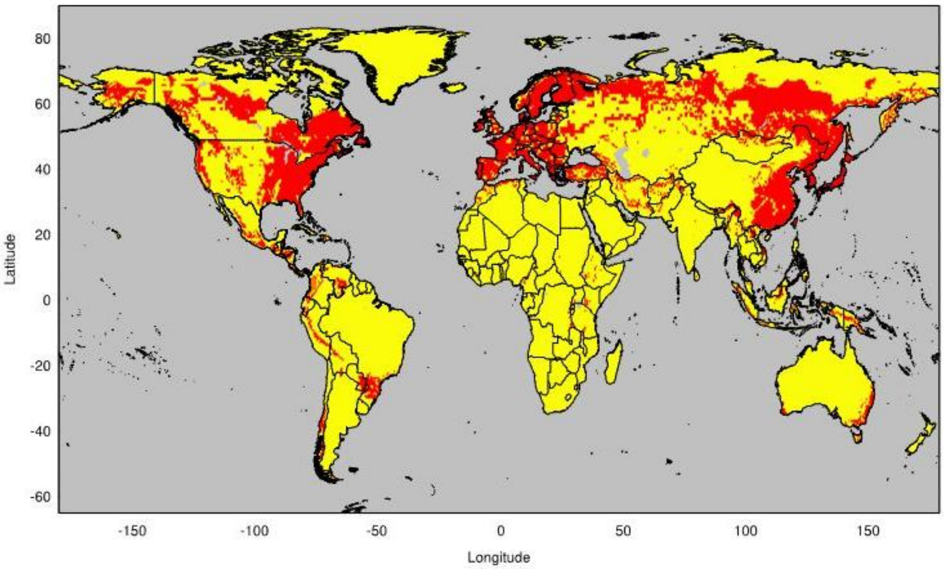

Figure 5. *Distribution of grid elements (red) that were identified as potential sites with alder based on the climate functions 1...3 (see also Figure 2). In this version of the model, restriction by vegetation types (see Table 3) and by soil units as found in Table 2 was applied. Yellow: grid elements were not identified as potential distribution area for alder.*

Potential distribution and migration of alder in 2100 and 2300

The absence of alders in the natural ecosystems can also cause an extreme decrease in nitrogen input by N_2 fixation of this plants group, and as a result can have gravely consequences for the nitrogen availability in soil of the areas. Therefore, it is important to model the migration of the N_2 fixing plants on global scale. To investigate this, the climate data of CCSM4 by driven four IPCC RCP scenarios up to 2300 were used. The rising of atmospheric CO_2 has enormous impact on climate change in the future. The using of climate data by driven different RCP scenarios enables to understand the effects of changed climate parameter (i.e. the T_{ann} and P_{ann}) by rising CO_2 on plant migration (in this study for alders). An increase in CO_2 in the atmosphere can also influence plant distribution by e.g. CO_2 fertilization, CO_2 partial pressure, water use efficiency (Johnson et al., 1993; Collatz et al., 1998). In this study, this type of impacts from CO_2 on the alder distribution was not considered.

For this step of the study, it was assumed that the soil unit and the potential natural vegetation groups of a grid element will not be changed in 2100 and 2300. The migration of alder species for those two prediction periods by using the ADM was shown in the *Figures 6* and 7. The results show that the alders can extend its distribution northwards. Especially the alder species may be frequently occurring furthermore in Northern Russia and Alaska at all scenarios of the climate models (see the blue areas in *Figure 6*). Few grid elements in Norway, Finland, the US and Canada may also additionally to be suited for the alder distribution in all scenarios in 2100. On the other hand, a range of grid elements close to coast in Europe, Southern US and Southern China may not have proper conditions anymore for alder distribution in 2300 (see the red areas in *Figure 7*). It is further to see that most of the grid elements in Africa,

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Indonesia and middle and south America may be eliminated for the alder distribution by all scenarios of the climate models in 2100 and 2300.

The prediction of potential migration of *Alnus spp*. by using the climate parameter of four RCP scenarios shown differences on global scale for the two projection periods (2100 and 2300).

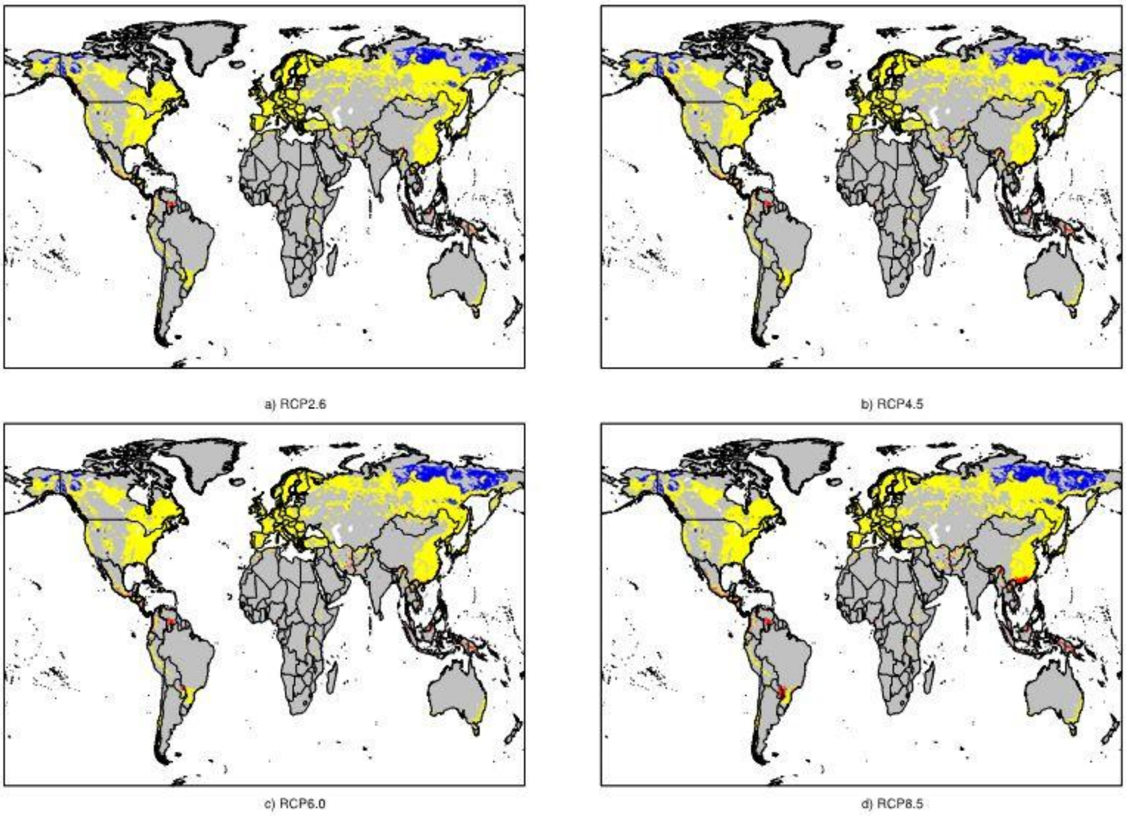

Figure 6. *Distribution of grid elements which were identified as potential sites for alder distribution based on the "All" method in ADM by using the climate data from CCSM4 data model which was driven by four RCPs emission scenarios. The colour "yellow" represents the potential distribution areas both present and in 2100, where the colour "red" shows the grid elements with present potential distribution but not in 2100, and also the colour "blue" the grid elements with potential distribution in 2100 but not present.*

The results indicated that only a change in two climate parameters (i.e. T_{ann} and P_{ann}) can affect the existence and distribution of plants in terrestrial ecosystems in the end of the 21th century. The validation of the methods also pointed out that the changes of soil types and vegetation compositions have enormous influences on the distribution of alders, and this should be considered in modelling studies about plant distribution.

The changes of the climate parameters within the four RCP scenarios have quite similar impacts on the alder distribution at global level in 2100 (see *Figure 6 a, b, c* and *d*). In 2300, the alder distribution was more affected by the change of the climate parameters in RCP8.5 scenario in the tropical and sub-tropical regions than other three RCP scenarios (see *Figure 7-d*).

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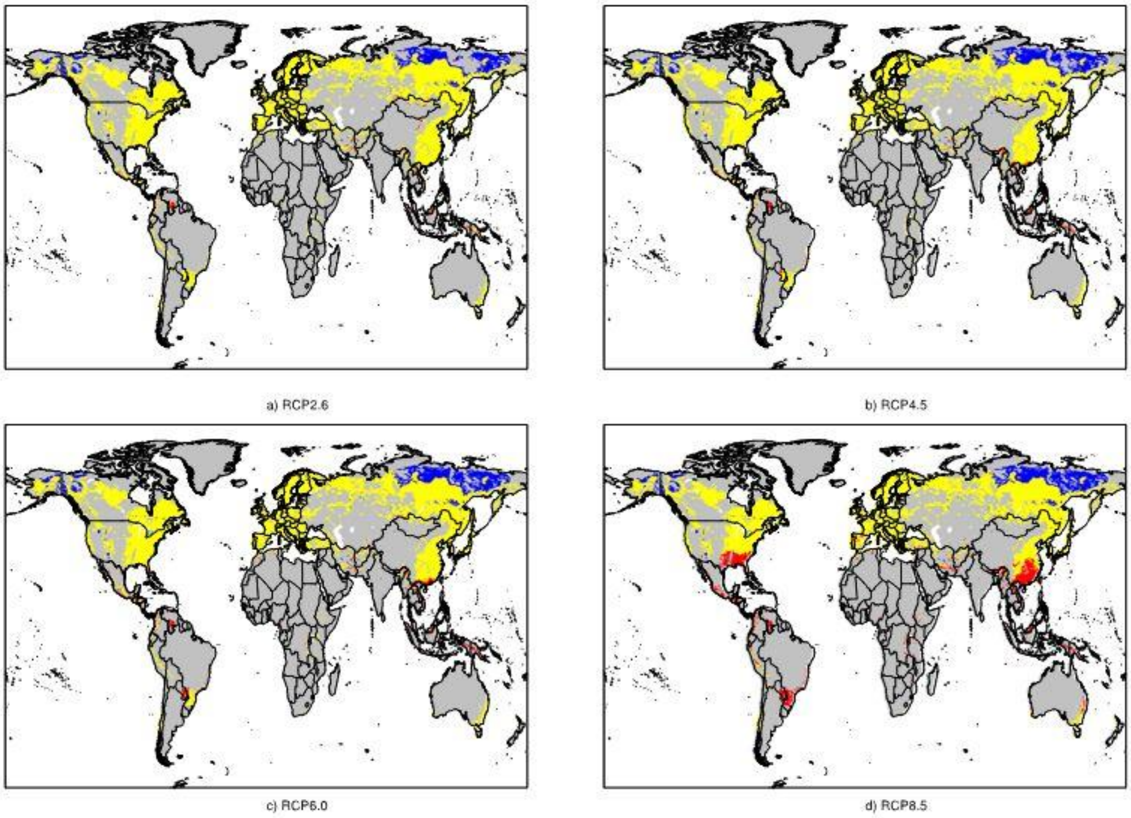

Figure 7. *Distribution of grid elements which were identified as potential sites for alder distribution based on the "All" method in ADM by using the climate data from CCSM4 data model which was driven by four RCPs emissions scenarios. The colour "yellow" represents the potential distribution areas both present and in 2100, where the colour "red" shows the grid elements with present potential distribution but not in 2300, and also the colour "blue" the grid elements with potential distribution in 2300 but not present.*

Discussion

The records for the distribution of the world's vegetation types was started by foundations of plant geography ca. 200 years ago (Humboldt, 1807). Also at the beginning of 18th century the scientist started to investigate about the potential effect of climate on plant distribution (Schouw, 1823; Meyen, 1846). Nowadays, numerous models use the climate conditions for the prediction of plant species distribution, and for the modelling of phenological processes of plants (Prentice et al., 1992; Brovkin et al., 1997; Smith et al., 2001; Skjøth et al., 2008; Sakalli and Simpson, 2012). Lantz et al. (2010) investigated the regional temperature impacts on the *Alnus viridis* subsp. *fruticosa* (green alder) patch dynamics and plant community (Lantz et al., 2010). They found out that the regional temperature influence the cover, growth, reproduction and age distributions of the green alder. Martínez-Meyer and Peterson (2006) worked on niche models to determine the distribution of eight taxa including *A. incana* and *A. viridis* in North America by using pollen distribution data on present day, and climate data from the Palaeoclimate Modelling Intercomparison Project in Last Glacial Maximum (LGM). They found a similar temperature-precipitation demand for the distribution of *A. incana* (see *Figure 3* in the paper). The using of only climate

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parameter in ADM predicted the distribution of all alder species almost in whole Australia, Middle and South Africa, where there is no or very poor record about the distribution of alder (see *Appendix Figure 1*). Also, the statistical analysis of the “Clim” methods has the poorest correlation in the 20 countries with high density records about the alder distribution. Sykes et al. (1996) developed a bioclimatic model based on climatic geography of the European plants to predict the distribution of northern Europe’s dominant trees including the alder species *A. incana*. They used the bioclimatic factors as winter cold tolerance, summer heat and winter cold requirements, and drought tolerance (soil moisture) of the species. Although the bioclimatic model supplied quite good result for the *A. incana* distribution, it also showed that the distribution of plants was not only depending on the climate factors but also the soil conditions could play a crucial role in the prediction. Like most of the models, the bioclimatic model could be used for prediction of the distribution of specific alder species (not for all species of genus *Alnus*) on regional scales and needs predefined parameter for each species. Compared to the bioclimatic model, the ADM has the advantage to predict the distribution of all alder species on global and regional scale.

Since, the selected climate factors were alone inadequately to predict the distributions of alder species, and it is well known that the components of each plant community are influenced by soil units, and the alders prefer some specific soil conditions for the occurring in a natural ecosystems (Bean, 1989; Wheeler and Miller, 1990; Claessens et al., 2010), the soil units were used as additionally determinant to the selected climate factors for the modelling of potential alder distribution areas. The addition of the soil units to the “Clim” method showed its impacts mostly in East Europe, Australia, Central and South Africa as well as in North America. But in comparison to the distribution Map from GBIF and the literature data, quite a lot areas were still selected as potential distribution locations for the alder species in that areas (see *Figure 4* and *Appendix Figure 2*). Also, the statistical analysis between the observed and predicted data in the 20 countries resulted a rare correlation (see *Figure 3b*).

In the natural ecosystems plants are living in a species compositions which are called plant communities (Schmithüsen, 1968). Each plant species belongs to a community and is related to other species of the community (Breckle, 2002). Therefore, the relations of the species in plant communities, and the using of the relations were quite important in modelling of distribution of plant species. Woodward and Williams (1987) investigated the effect of climate on plant distribution on global and local scales. Their predictions of the distribution of the vegetation were based on temperature, precipitation and annual water balance of the distribution areas. They enhanced also that the climate conditions are not sufficiently for the modelling of the distribution of vegetation or species, and in such modelling studies, the population dynamics (plant–plant interactions) should be also considered. Therefore, the potential natural vegetation groups after Schmithüsen (1976) were used as additional determinant to the “Clim method” for the prediction of the potential distribution areas for alder species. Although the statistical of the results in the 20 countries showed quite good correlation with the observed data from the GBIF database (see *Figure 3c*), on the global scale, the comparing of the results (see *Appendix Figure 3*) with the distribution map from GBIF database (see *Figure 4*) presented noticeable differences in East Europe, in Canada, Southeast Australia and America. In some local studies, the scientist tried to find out the interspecific relationships between plant species in plant communities, and the

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relationships between the dispersal of the species and the environmental, biological, and geological factors. Jones et al. (2008); Flinn et al. (2010); Aiba et al. (2012); Lin et al. (2013) pointed out that plant dispersal is not depending on environmental factors. They found a poor correlation between the environmental factors and plant dispersal. On the other hand, they also did not use the combination of annual average temperature and sum of the precipitation as determinant in their studies. The used parameters (Wind, NO3, soil humus content etc.) were also dynamic parameters, which can have strong seasonality. The ADM considered the average of 30 years of the climate data (1961–1990). That eliminated the uncertainties regarding to the dynamic seasonality of climate parameters.

Although, only 6% of the alder distribution can be explained by using the climate data in this study (see *Figure 3*), results of this study showed that the distribution of plant species was not only depending on the climate factors but also on the soil types, and the vegetation units should be considered together. The additions of soil units and potential natural vegetation groups to the "Clim" method pointed out that both determinants can influence the prediction of the potential distribution areas of alder species in different regions. Therefore, all determinants were merged in one method for the modelling of the potential distribution areas of alder species. The "All" method of the ADM shows a new kind of modelling issue for plant distribution. The statistical analysis of the "All" method results showed quite good correlation and the best value of index of agreement as well as the lowest *MAE* (see *Figure 3d*). The predicted potential distribution areas for alder species using the "All" method was presented in the *Figure 5*. In comparison to the potential distribution maps of the "Clim", "Soil" and "Veg" methods, there is a further improvement of the predicted distribution especially in Central and Eastern Asia, and in America. But there are still differences between the observed and predicted distribution areas in Asia, Africa, Southeast Australia and America. It is well known that alder grows well on acid soils and its growth can be restricted under the alkaline or neutral conditions. "Lithosols" are typical soil unit in temperate climate zone under coniferous forests, and the "Camsbisols" are well represented in boreal and temperate regions. The two soil units are well represented in Russia and known as acid soils. Suitable climate conditions and vegetation groups make possible to distribute the alders in large areas in Russia. Murai (1968) published the potential distribution areas of alder species (*A. viridis* and *A. crispa*) in Russia in a vegetation map (Murai, 1968). It shows that the distribution of *A. viridis* and *A. crispa* stretches in most vegetation zones of Russia. Also, Kajba and Gracan (2003) illustrated a map for the distribution of *A. glutinosa* in Europe (Kajba and Gracan, 2003). It showed that *A. glutinosa* also distribute in several locations in Russia. These maps confirm that the results of ADM prediction for Russia are acceptable. Furthermore, the GBIF database does not indicate the absence of alders. Therefore, grid elements with no data records may indicate either the absence of alders or the absence of observations. Because of the suitable climate, soil, and vegetation conditions, it is highly probable that alders can distribute in these areas (Czerepanov, 1995). Globally, ADM may provide better results for the distribution of this genus. The discussion of this results also shows the importance to improve the GBIF database for validation of such model results. In China, the alder distribution was recorded in only three grid elements (see *Table 4*). But "All" method based ADM predicted the distribution in 1376 grid elements more than the GBIF database. Likewise, it is well known that *A. nepalensiss* distributes in moist, cool, subtropical monsoon climates with a dry season of 4–8 months in Guangxi,

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Guizhou, SW Sichuan, Xizang, Yunnan of China (Furlow, 1979; Sharma and Ambasht, 1991; Chen, 1994; Jackson, 1994; Dorthe, 2000; Chen and Li, 2004). It also shows that the prediction of ADM for alder distribution is more reliable than GBIF database in China. Furthermore, some regions in Central Africa and southeast Australia still remain. The regions in Central Africa are known to be suitable for alder cultivation, however, no natural occurrence of alders is recorded in the areas. Such plantations of alder species are also recorded in African highlands (Wajja-Musukwe et al., 2008; Muthuri et al., 2009; Siriri et al, 2013). Niang et al. (1996) published data about the adapted alder species (*A. acuminata* HBK) to the highlands of Rwanda in Central Africa. The average annual rainfall is 1500 mm and the annual mean temperature is 14.6 ºC in the study site. The values of the climate parameters are in the climate field (see *Figure 2*). The dominant soil unit is a “Podzols” and the vegetation group is a “tropical forests of higher elevation”. Both the soil unit and the vegetation type are presented in the 308 evaluation’s data (see *Tables 2* and *3*). This result shows that the alder species can well distribute in some areas of Central Africa, and the prediction of ADM can be right in Central Africa. In southeast Australia, 8 records in totally 3 grid elements were recorded in GBIF data base (see Table 4). But the model results shown that alders potentially can distribute in 157 grid elements in this area. The T_{ann} and P_{ann} values in the regions range 8–15 ºC and 500–1100 mm, respectively. The dominant vegetation unit is “Laurel mountain forests” as well as “Laurel forest w. conf. trees”, and “Luvisols” are the most recorded soil types in southeast Australia. The values of the climate parameter in the 157 grid elements are found in the climate matrix field in the *Figure 2* as well as the vegetation units and the soil types are appropriate units and types for a potential alder distribution. Therefore, it is quite possible that the alders can have larger distribution than as recorded in GBIF database for southeast Australia. Hnatiuk (1990) also recorded an alder species (*A. glutinosa*) in Australia, and indicated that four related species have also naturalized in Australia. But, there is no information about the distribution locations of the alders in his study.

A visible and an important difference between the predicted and observed alder distribution is to see in South American lowland and Araucaria forests in Brazil and Paraguay (see *Figure 5*). The ADM show the potential alder distribution in 168 of 1 653 grid elements in Brazil and in 40 of 143 in Paraguay where the GBIF database does not include data records about the alder distribution. But, Ledru et al. (2007) and Behring (1997) published data about the pollen distribution of some alder species in Araucaria forests in South Brazil. Also, Marchant et al. (2002) presented data about pollen distribution of alder in several Middle and South American countries. They found alder pollen in gallery forests and forests with *Quercus–Pinus* species. These pollen data show that alder species have distributed in the regions of South America with suitable climate conditions, soil units and vegetation groups. But, data records about current occurrence of alders with coordinate data are still needed in the regions for a reliable comparison of the model results.

The pollen records of the alder species in some areas, where the alders currently do not represent show that the alders have potential for migration. Van Minnen et al. (2000) reported that the alders need between 50 and 200 years to change its distribution areas due to the climate change. The migration of nitrogen fixers will certainly influence the natural nitrogen fixation in the ecosystems. Esser et al. (2011) showed the effect of nitrogen fixation on carbon biogeochemical cycle by switching off and on the nitrogen fixation fluxes in the nitrogen carbon interaction model (NCIM) (see model scenarios in

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Esser et al., 2011). They presented a three times carbon storage with a nitrogen availability than without in the biosphere. Since a migration of N_2-fixing plants changes the amount of available nitrogen in soil for soil microorganisms and plants, as important N_2-fixing group, the investigation of the spatial and temporal alder migration is quite important.

The using climate parameters from different climate models and scenarios gives important indications of climate change effects on alder distribution and migration in the future. As it presented in *Figure 1*, CCSM4 provides variously T_{ann} and P_{ann} by using four IPCC RCP scenarios. There is quite difference between the values of the T_{ann} as well as P_{ann} of the models, and the difference between the four scenarios of the models are quite large (ca. 16 ºC for T_{ann} and ca. 250 mm for P_{ann} in 2300). Though, the effects of the climate parameter of the models, and scenarios on the alder distribution on global scale are quite similar (see *Figure 7*). There is unfortunately no similar study to compare the results of the ADM by using values of the climate parameter from the four IPCC RCP scenarios of a GCM model. Therefore, it is assumed that the prediction of the potential alder distribution (migration) in the future is quite reasonable according to the results of this study.

Conclusion

In this paper, a new methodology for predicting of potential distribution of alder species on global scale is presented. The new methodology of ADM gives the scientist the possibility to understand the climatological and ecological requirements of alder species to distribute in natural areas and the opportunity to implement a simple method to predict the potential distribution locations of the alder species on each resolution. The using simplified approaches as in "All" method of the model allows the scientist to understand the functionality of plant distribution by considering the environmental and geological factors. The model shows that combine effect of the all three parameters (i.e. climate, soil, vegetation) is the predictor for the identification of potential habitats for the alders. Climate alone may not predict the range of alders correctly. By using soil units and potential natural vegetation groups as additional predictors, the identification of potential alder sites is much closer to the presented distribution map of GBIF database. In this paper, the temporal and spatial change of alder distribution was modeled by using the climate variables from the CCSM4, which was driven with four RCP scenarios (RCP2.6, RCP4.5, RCP6.0, RCP8.5) up to 2300. The using of climate variables should give an overview of the sensitivity of the methodology for modelling of plant distribution according to climate models. The using of the climate variables of four RCP scenarios in this study allows the scientists the investigation of the sensitivity of plant distribution by considering the different emission scenarios on global scale up to 2100 and 2300, respectively. Because of the missing dynamic vegetation data and dynamic soil unit data for 2300, only the climate parameters were dynamically changed in the ADM for predicting the potential distribution of alder species in 2300. The results of the ADM show that numerous regions in Northern Hemisphere will get the suitable conditions for the migration of alder species. But also, a lot of grid elements in Southern Hemisphere will not be suitable for alder occurrence.

Although clearly dynamic datasets for soil units and vegetation groups are needed for a testimonial evidence, the simple requirements of the ADM methodology might make it suitable for use in other biogeochemical models and other modelling systems.

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APPENDIX

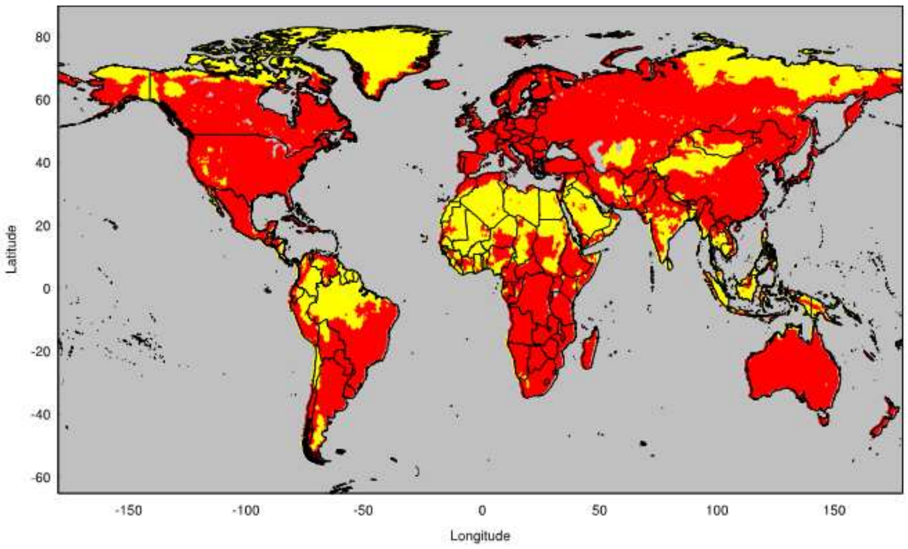

Figure A1. *Distribution of grid elements (red) which were identified as potential sites with alder based on the climate functions 1…3 (see Fig. 3). Yellow: grid elements were not identified as potential distribution area for alder.*

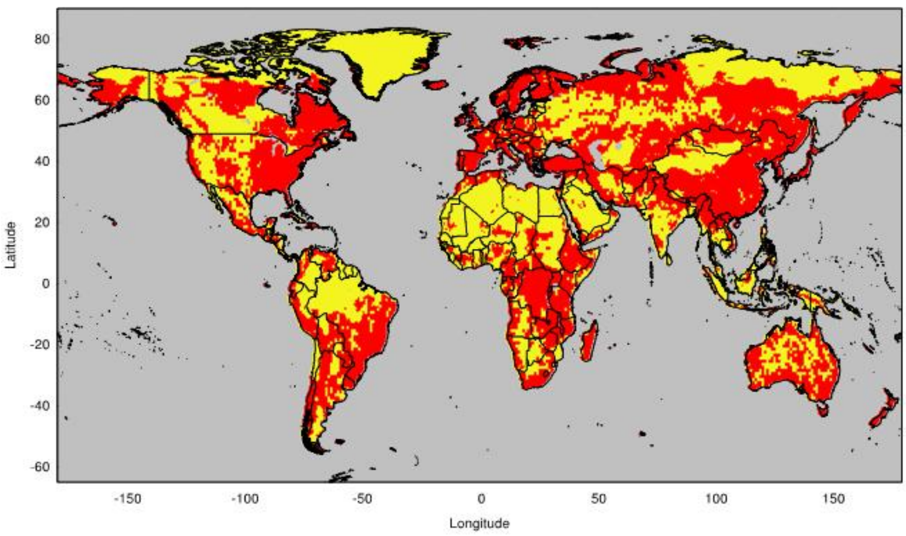

Figure A2. *Distribution of grid elements (red) which were identified as potential sites with alder based on the climate functions 1…3 (see Fig. 3). In this version of the model, restriction by soil units as found in Tab. 1 was applied. Yellow: grid elements were not identified as potential distribution area for alder.*

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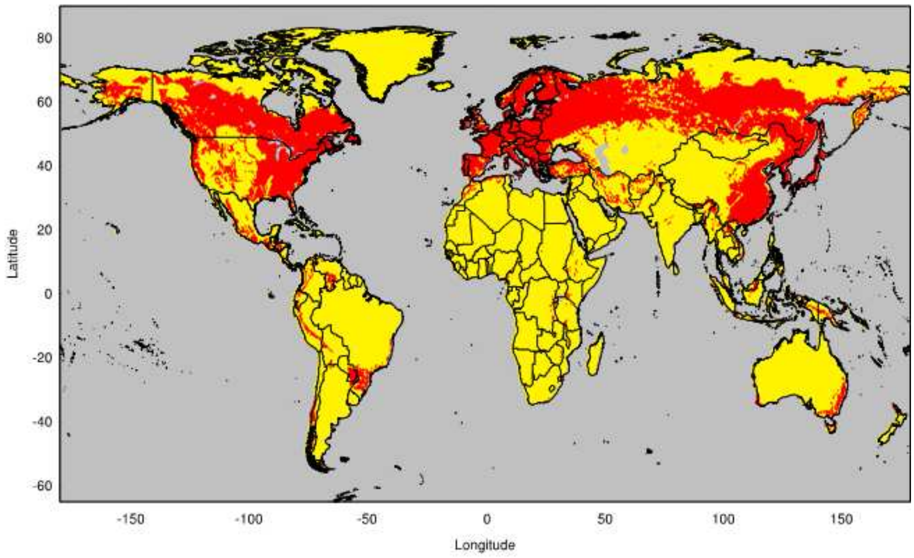

Figure A3. *Distribution of grid elements (red) which were identified as potential sites with alder based on the climate functions 1…3 (see Fig. 3). In this version of the model, restriction by vegetation types (see Tab. 2) was applied. Yellow: grid elements were not identified as potential distribution area for alder.*

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ECOLOGICAL AND ENVIRONMENTAL RISK ASSESSMENT IN THE NANOMATERIALS PRODUCTION

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Abstract. In this study, the spray pyrolysis and hydrothermal production processes of nanostructured zinc oxide were compared in terms of health and environmental risk on a semi-industrial scale with a capacity of 2000 kg/year. For this, environmental release and human exposure to identified sources of pollution in each production process were modeled using the Chemical Screening Tool for Exposure and Environmental Release (ChemSTEER) software. Spray pyrolysis and hydrothermal were compared pair-wisely by analytic hierarchy process (AHP), which is a multi-criteria decision-making model using health and environmental indicators. According to AHP results and box plot chart, hydrothermal process leads to a higher health and environmental median inverse risk compared with spray pyrolysis. Thus the synthesis of nanostructured zinc oxide by hydrothermal process is safer than spray pyrolysis. Due to the growing development of the various methods of the nanomaterials, we need to apply the safer methods and to have the least ecological risks in the production of the nanomaterials. These findings could be helpful for modifying the operation and reducing the ecological potential risks to scale up the existing pilot plant to an industrial unit.
Keywords: *ecological aspects*, *environmental release, nanotechnology, process design, analytic hierarchy process*

Introduction

Environment and ecological risk assessment

The word “Ecology” literally means the mutual effects of the environment on the living organisms, and the living organisms on the environments along with interrelationships between organisms. (Michener, 2006) A branch of the ecology is to understand the relationship between man and the environment and their consequences on the environment. The experts have hardly emphasized on the effect of the human activities on the features of the ecosystem up to now. However, to figure out the structural changes in the ecosystem, we need to have a comprehensive understanding of the stressors and their impacts on the environment and the ecosystem. (Jounston et al., 2015) It should be noted that the chemical pollution is one of the stressors that we can find everywhere, and it can make some changes in the process of the ecosystem. (Mc Mahon et al., 2012). Pollutions from many sources in various formats can go to the

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ecosystem, besides they can cause many environmental risks. Therefore, the environmental risk assessment of the chemical contamination can be a step towards a reduction of the dangers and the protection of the environment. The risk assessment is a rational way to determine the size of the quantitative and qualitative hazards, and the possible consequences of the hazards on people, materials, equipment and the environment (ISO/IEC, 1998). It has four stages.

- The description and identification of the hazards.
- The exposure assessment (e.g. the emission or environmental fate).
- The risk assessment.
- The risk management (Ostertag and Husing, 2008). The results of the risk assessment can effectively lead to changes in the environmental management policies. (Vall-llosera and Sol, 2009). We consider the nanotechnology as an applicable technology in recent decades. This technology comes from the convergence of physics, chemistry and biology.

Some studies consider nanotechnology the next industrial revolution (Scrinis and Miller, 2006), as it is changing other industries, including health, environment, agriculture, energy, materials, and communication sciences (Rickerbya and Morrison, 2007).

Nanotechnology has been able to make some significant changes in various industries, for instance, using carbon nanotubes which have been able to upgrade the mechanical properties of tires, or the nanoparticles are used as the catalysts for the petrochemical industry. Nevertheless, the particles have the potential to cause the ecological hazards. Moreover, several factors can make the nanoparticles go to the environment through the air, soil and groundwater. They can be a threat to the organisms in the environment or vicinity of the environment. The key aspects of the risk assessment in the field of the nanomaterials are the identification of the physical and chemical properties of nanomaterials, the identification of the environmental hazards, the identification of the hazards, and how humans face with ecosystem risks and their effects (Colvin, 2003; Maynard, 2006). The results of the risk assessment are the important components to ensure various industries in the safety of their industry (Drobne, 2007). In this way, we can determine the efficiency of the inventory controlling procedures; moreover, we can prepare some valuable data for making decision on the reduction of the risks, hazards, upgrading the control systems and planning to respond to them.

Ecological impact and nano technology

Nanotechnology provides the ability to fabricate smaller materials with a significantly reduced volume of active materials, a high surface area to volume ratio, and increased performance (Moore, 2006; Gregory, 2010).

The high-level reactivity of the nanomaterials can be a threat to humans and the environment. This increased reactivity gets the nanoparticles to have an uncontrollable and unpredictable high potential in the reaction with many molecules found in the nature, cells and organisms. On the other hand, the very small size of the nanoparticles reinforces the hypothesis that particles can pass through the immune system. Some inhaled particles can enter the lungs; consequently, they can enter the bloodstream. (Moghimi and Hunter, 2001)

Due to the increasing consumption of these materials industries, for instance, the nanoparticles such as titanium dioxide are some of the air pollutants which can cause

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inflammation and lesions of the mucous, and accumulate in the lungs (Moghimi and Hunter, 2001). In addition, the nanoparticles of the titanium dioxide and zinc oxide in sunscreens can cause free radicals in skin cells and damage DNA. Damaging to DNA can also make some changes in the structure of the proteins and their functions; subsequently, they can lead to the cell cancer, the metastasis and tumors (Poirier, 2004). Titanium dioxide nanoparticles have some other applications including cleaning the environment, soil, water and air. Hence, these particles can easily enter the environment, and they can enter to the food chain through vegetarian and fish, owing to it, they can spread out in organisms in ecosystem. The processed carbon nanoparticles gluten causes the depletion and the oxidation in fish's brains and changes in the functions of genes. The substance can also be transmitted through the soil, as a result of it, earthworms can absorb these substances, and then they enter to the food chain (Oberdorster et al., 2004). The miners and workers exposed to the nanoparticles of quartz (the carcinogenic materials) are susceptible to the dangerous diseases. The researches show that the impacts of the carbon nanotubes are more toxic than the quartz dusts on mice's lungs in the laboratories (Lam et al., 2004). The high surface reactivity of quartz and carbon nanoparticles can make free radicals; consequently, it can cause the oxidation of the white blood cells and immune cells.

The formation of carbon nanotubes and nanocrystals are accumulated in the air.

Nanocrystals composed of the carbon particles are more dangerous than the individuals not the accumulated ones to the living organisms. The researches also show that the unprocessed carbon nanotubes, which are either displaced or aroused enough, can form the dust particles (Maynard et al., 2004). Carrying the carbon nanotubes is somehow hazardous to the health. Furthermore, the factories which are either the manufacturers or the consumers of the carbon nanotubes-based products endanger the health of workers (Oberdorster et al., 2004).

Increasing use of nanotechnology and nanomaterials has significantly increased exposure of workers to nanomaterials, increasing threats to employee health and to the environment (Savolainen et al., 2010). This necessitates the need for increased global research on the use of nanotechnology to combat unsanitary living conditions (Nowack and Bacheli, 2007).

The unclear adverse effects of nanotechnology on humans and the environment (Ostertag and Husing, 2008) have led many organizations in the world, such as the national institute of health, national nanotechnology initiative, nanomedicine roadmap initiative in the USA, center of nanotechnology, institute of nanotechnology, nanoscale science and nanotechnology group, manufactory engineering center, and other similar organizations in Europe to invest heavily in nanotechnology research.

This study is also done to promote research in the field of nanotechnology and nanoparticles as an emerging technology. Studies on the ecological effects of the chemicals often occur in the early stages of the materials consumption and their disposal. In addition to the ecological assessments in the consumption and disposal, we can do these studies in the stages of production of materials. In this case, the manufacturing processes and the ecological effects are less developed by the result. Many studies have been done about the assessment of the biological environment and health during the production process of a substance. In 2010a, Hasim and Hurmeh evaluated and compared the methyl methacrylate production based on the health indices through the six stages. In the same year (2010b), they introduce a method known as the health quotient index (HQI) in another study. The HQI method presents a simple

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approach to assess the people's health which some hazardous factors may expose their health; moreover, this method can present the ranking of the manufacturing process risks. Their method has been tested in six different- production process of the methyl methacrylate.

These authors also estimated the chemical concentration of fugitive emissions during chemical process design (Hassim, Perez and Hurme, 2010). Similar studies have been conducted by Srinivasan and Trong (2010). Gupta and Edward (2003) introduced some indices such as the Inherent Safety Design index. In another study, Rahman, Heikkila and Hurme (2005) compared inherent safety index methods and discussed their properties and limitations. A case study of methyl methacrylate process routes was conducted in 2005. In the same year, Faisal khan and Paul Amyotte presented the conceptual framework of the inherent safety index. Risza and Azmi (2010) proposed a methodology that integrated the hazard review technique with inherent safety, which is useful for identifying risks at an early stage of the production process

Objectives

This study aims to introduce a suitable method of zinc oxide nanostructure production with regard to health and environmental parameters.

In this study, spray pyrolysis and hydrothermal processes for producing nanostructured zinc oxide were compared under normal operating conditions with regard to health and environmental risks.

The high strength of zinc oxide under the harsh conditions of industrial processes and its high activity at the nanoscale (Rajendran et al., 2010) in comparison to the microscale (Zhang et al., 2009), low toxicity, good thermal stability, good oxidation resistibility, good biocompatibility, large specific surface area and high electron mobility (Pan and Huang, 2011) suggests the importance of using the material in different industries. The high consumption rate of zinc oxide in various industries on one hand, and the ability to produce zinc from domestic sources on the other hand, are encouraging factors for the study on health and environmental risks of producing nanostructured zinc oxide.

Spray pyrolysis is based on the liquid atomization technique (Madler, 2004). It is an economical, easy, and continuous technique *(*Charp et al., 2015*)*. In the spray pyrolysis process, a solution of zinc salt [Zn $(NO_3)_2.6H_2O$] are placed under a pressure and temperature of 20 bar and 80 °C, respectively. The decomposed droplet as resulting mixture was pumped as feedstock into a spray dryer reactor at a temperature of 500-800 °C (Ghaffarian et al., 2011). The product was subsequently fed into a cyclone to separate the particles based on their sizes. A scrubber and caustic soda were used to neutralize NO_x emitted from filtering process.

The hydrothermal method is based on crystal synthesis at temperatures above 100°C and pressures above 1 atom (Hayesh and Hakuta, 2010). It uses simple equipment, is catalyst free and low cost (Aneesh, Vanaja and Javaraj, 2007), and constitutes a batch method. In this study the crystals of zincsalt [$Zn(NO_3)_2.6H_2O$] is used. Leached solution from reactor will be cold rapidly and then filtered.

In this study, we compared, for the first time, the spray pyrolysis and hydrothermal production processes of nanostructured zinc oxide under normal operating conditions in terms of health and environmental risks at Iran's Research Institute of Petroleum Industry (RIPI) in 2015.

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Environmental release and the exposure level to chemicals during each of the processes with the same production capacity was determined separately for each pollutant sources. Subsequently, spray pyrolysis and hydrothermal processes were compared pair-wisely using analytical hierarchy process based on respiratory harms, skin harms, and environmental damages such as air, water, and soil pollution to determine the optimal method in terms of health and environmental risks.

Materials and methods

As already mentioned this study aims to introduce a suitable method of zinc oxide nanostructure production with regard to health and environmental parameters. For this purpose, pollution sources in zinc oxide nanostructure spray pyrolysis and hydrothermal processes were identified. The Chemical Screening Tool for Exposure and Environmental Release (ChemSTEER) was used to model the release of possible pollutants and the amount of human exposure in each process.

ChemSTEER software, developed by the United States American Environmental Protection Agency (US EPA) was used to model the release of possible pollutants and the degree of human exposure in each process. The software is capable of estimating the extent of respiration and skin contact with chemicals during industrial and commercial production processes. It can also estimate the amount of chemicals released into the environment during industrial production (EPA, 2014).

According to the results of ChemSTEER, the spray pyrolysis and hydrothermal process were compared pairwise using the analytic hierarchy process (AHP) in Expert Choice based on five criteria. Respiratory and skin damage as the health subcriteria and air, water, and soil pollution as the environmental subcriteria to investigate suitable zinc oxide nanostructure production methods with regard to the environment and health. Criteria mentioned are the most important criteria in health (Hassim and Hurme, 2010c) and environmental assessment (Kweku et al., 2008; Som et al., 2011*)* which have frequently been used by researchers.

AHP method was performed by weighting of criteria and prioritizing of alternatives using eigenvector technique. This is one of the most comprehensive systems designed for decision making with multiple criteria by which it is possible to formulate the problem as a hierarchy. It also offers the possibility of involving qualitative and quantitative criteria in decision making (Saaty, 1986, 2008).

According to AHP results the box plot diagram of inverse risk has been drawn, and the optimal production process of nanostructured zinc oxide was introduced

Results

As mentioned earlier, each of the two processes has been studied from health and environmental aspects. For this, following the investigation of spray pyrolysis and hydrothermal processes and identification of pollution sources in each process, skin and respiratory exposure to chemicals released into the environment were modeled by ChemSTEER software.

The pollution sources of spray pyrolysis under normal operating conditions includes NO_x fumes from the feeder, spray dryer, and scrubber and sodium nitrate solid waste from filtration. Pollution sources of hydrothermal process under normal operating

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conditions include NOx fumes from the reactor and waste water include sodium nitrate and different kind of solvent.

In this study, the emission sources and human exposure of spray pyrolysis and hydrothermal methods used in two semi-scale nanostructured zinc oxide production processes with a capacity of 2000 kg/year were modeled by ChemSTEER software. To run this software, the chemical properties of primary substances, including their vapor pressure, molecular weight, density, and solubility in water, were required. Next, operation parameters and polluting sources or activities were determined. ChemSTEER has provided default models. The estimation of the environmental release and chemical exposure occurs according to the operation process and polluting sources. Spray pyrolysis is a continuous process; hydrothermal is a batch process. The duration is eight hours for the former and one hour for the latter. Production per process of each is 100kg; the number of individuals exposed to the emissions in each working shift is 5 people.

According to the results, the average daily exposure resulting from respiratory contact with NO_x emitted from the feeder, spray dryer, and scrubber in the spray pyrolysis process are 0.00012, 0.00096, and 0.00004 mg/kg/d, respectively. While the average daily exposures from inhalation and skin contact with the waste storage tank in the spray pyrolysis process containing sodium nitrate are 0.039 and 0.8088 mg/kg/d, respectively. The environmental release of NO_x emitted from the feeder, spray dryer and scrubber in the spray pyrolysis process are 0.012, 0.1, 0.004 μg/m³, respectively. Moreover, the possible daily release rate of sodium nitrate from waste storage tank is 0.161 kg/day.

The average daily exposure resulting from respiratory contact with NO_x emitted from the reactor in the hydrothermal process is 0.02 mg/kg/d. While the average daily exposures skin contact with the waste water in the hydrothermal process containing sodium nitrate and different kind of solvent is 1.2 mg/kg/d. The environmental release of NOx emitted from reactor in the hydrothermal process is 0.16μg/m³.

Tables 1 and *2* present the results of respiratory and skin contact along with environmental release modeling for each of the pollution sources in spray pyrolysis and hydrothermal.

Table 1. *Inhalation and skin contact*

	Type of Contact	**Pollution Sources**	**Potential Dose Rate (mg/d)**	**Life Time Average Daily Dose (mg/kg/d)**	**Average Daily Dose (mg/kg/d)**	**Exposure Limit TWA (8 h)**	**Acute Potential Dose (mg/kg/d)**
Spray Pyrolysis Method	Inhalation	No_x emission from feeder	0.1546	0.000069	0.00012	5 ppm	0.0022
		No_x emission from spray dryer	1.2328	0.00055	0.00096		0.018
		No_x emission from scrubber	0.061	0.000027	0.000048		0.0009

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		Waste storage (sodium nitrate)	49.99	0.022	0.039	5 mg/m^3 or 1.438ppm	0.7142
	Skin	Waste storage	1033.23	0.4622	0.8088	5 mg/m^3 or 1.438ppm	14.7604
Hydrothermal	Inhalation	No_x emission	4.98	0.0022	0.02	5 ppm	0.56
	Skin	Waste water	202.45	0.091	1.2	-	22.4

Table 2. *Environmental release*

Method	Pollution Sources	Media	Daily Release Rate (kg/site-day)	Annual Release Rate (kg/year-all site)	Annual Concentration (μg/m³)	Annual Standard
Spray Pyrolysis	No_x emission from feeder	air	0.012	0.026	0.012	0.053 ppm (100 μg/m³)
	No_x emission from spray dryer	air	0.01	0.2	0.1	
	No_x emission from Scrubber	air	0.0005	0.01	0.004	
	waste storage (sodium nitrate)	soil	0.161	0	-	-
Hydrothermal	No_x emission from reactor	air	0.002	0.051	0.16	0.053 ppm (100 μg/m³) annual
	Waste water	soil/water	0.5	10	-	-

According to *Table 1*, the average daily exposure to NO_x and solid particles in a sodium nitrate waste storage tank during spray pyrolysis and the average daily exposure to NOx from reactor during the hydrothermal process are below the 8-hour occupational exposure limit. Also, the average daily exposure of skin contact to sodium nitrate during spray pyrolysis is 0.8088 mg/kg/d, which is below the 8-hour occupational exposure limit, regardless of the acute potential dose

According to *Table 2*, the emissions from feeder, spray dryer, scrubber during spray pyrolysis and reactor during hydrothermal process are below the standard.

To introduce a suitable method of zinc oxide nanostructure production with regard to health and environmental parameters, the spray pyrolysis and hydrothermal techniques were compared pair wisely using AHP in expert choice software based on respiratory harms, skin harms, air, water, and soil pollution according to the results of ChemSTEER. The criteria were weighted by averaging the scores in the structured questionnaires given by industrial and academic experts in environment, health, ecology, chemistry and nanotechnology.

The obtained results indicate that the hydrothermal process with 0.61 wt % has a lower health and environmental risk than the spray pyrolysis process with a 0.39 wt %.

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Thus, it can be concluded that the hydrothermal process would be a more health and environment- friendly process for the production of nanostructured zinc oxide (*Table 3*).

Table 3. *Pair wise comparison of spray pyrolysis and hydrothermal*

	Health criteria		**Environmental criteria**			**Final result**
	respiratory harms	**skin harms**	**air pollution**	**soil pollution**	**water pollution**	
Spray pyrolysis	0.125	0.5	0.25	0.5	0.5	0.39
Hydrothermal	0.875	0.5	0.75	0.5	0.5	0.61

Also the box plot diagram of inverse risk has been drawn according to AHP results (*Figure 1*). Box plot diagram indicated that hydrothermal process leads to a higher health and environmental median inverse risk compared with spray pyrolysis. Obviously, higher inverse risk leads to a safer health and environmental condition. Thus it can be concluded that the hydrothermal process with higher inverse risk would be a more health- and environment-friendly process for the production of nanostructured zinc oxide.

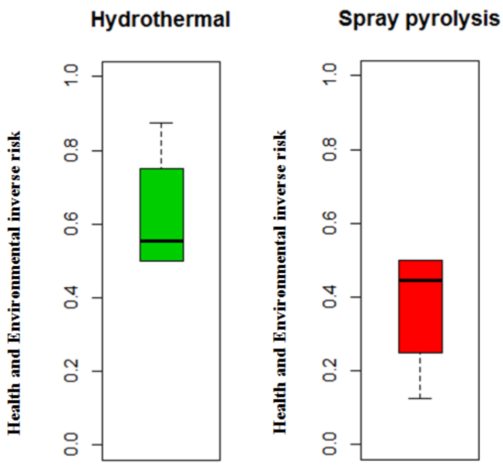

Figure 1. *Box plot diagram of inverse risk*

Discussion

Prior works have been carried out on the health and environmental impacts of nanotechnology (Gregory, 2010; Dreher, 2004; Moore, 2006; Steffi and Jurgen, 2007),

Studies on the ecological effects of the chemicals are often done in the early stages of material consumption and its disposal. In addition to the ecological assessments in the consumption and disposal, we need to study them in the production of materials. Thus, we can control the manufacturing processes and the ecological effects through the analysis of the results. several Studies on ecological and environmental risk assessment in production processes, especially during the early stages of the production process, except nano-materials, have also been conducted (Hassim and Hurme 2010a; Rahman et al., 2005; Srinivasan and Trong, 2008).

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What we can get from the similar surveys of the risk assessments of the chemicals in manufacturing processes is the lack of comprehensive studies on the nanomaterials production processes. Due to the growing development of the various methods of the nanomaterials and the development of the products based on nanoparticles, we need to apply the safer methods and to have the least ecological risks in the production of the nanomaterials. In this case, we are able to step in reducing the environmental risks associated with the development of this modern technology.

The above-mentioned results are based on health and environmental risk assessment of spray pyrolysis and hydrothermal synthesis of nanostructured zinc oxide on a semi-industrial scale. These findings could be helpful to mitigate ecological risk and scale up the existing pilot plant to an industrial unit with minimum environmental impacts.

The increasing use of nanoparticles as an attractive tool in the industry, as well as difficulties controlling the size and shape of nanomaterials, suggest the optimal method of nanomaterial production. This is despite the health and environmental consequences of nanomaterial production, which may be irreversible.

Studies on adverse health and environmental effects during the early stages of technology development and industrialization could effectively mitigate ecological risks. Process modification, substitution of hazardous chemicals with safer compounds, and presenting risk mitigation strategies are important topics, especially in nanomaterial production processes. These studies could be useful in identifying and reducing risks during the initial stages of the development process.

To this end, this study was conducted to identify the health and environmental risks of nanostructured zinc oxide production processes, which have received relatively little attention. The distinguishing feature of this study is to analyze the risks of nanomaterial production on a semi-industrial scale for the first time.

In this study the emission sources and human exposure of spray pyrolysis and hydrothermal methods used in two semi-scale nanostructured zinc oxide production processes were modeled by ChemSTEER software. Using ChemSTEER, spray pyrolysis, and hydrothermal techniques were compared pair-wise using AHP on respiratory harm, skin harms, air, water, and soil pollution indices.

According to the result the hydrothermal process with 0.61 wt % has a lower health and environmental risk than the spray pyrolysis process with a 0.39 wt %, so the production of nanostructured zinc oxide by hydrothermal processes is less risky than spray pyrolysis from health and environmental viewpoints.

On the one hand there is the management of the environmental and ecological risks; on the other hand we need to take some necessary measures to improve the performance of the method in the early stages, because the development of the production processes could be much more efficient and cheaper.

It is therefore recommended that–to develop a safer industry and to prevent irreparable events and environmental impact–further research is required on this topic**.**

Evaluating the other processes in the production of nano-materials disposal, we can use either other models such as fuzzy models of high accuracy or the neural network models which are capable of making new structures for the information processing system. We can compare the results of the hierarchical models which are accessible, functional and reliable by them. It is clear that these studies could help both to develop more secure industries and to prevent the devastating effects of the ecological and environmental factors.

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MULTI-CRITERIA DECISION MAKING FOR SUSTAINABILITY EVALUATION IN URBAN AREAS: A CASE STUDY FOR KERMANSHAH CITY, IRAN

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Abstract. Sustainable development refers to interactions between different aspects of economic, social and environmental features that are designed to improve the quality of human life. The aim of this study is to evaluate urban sustainability in different urban areas of the Kermanshah city of Iran. An important city in western Iran, Kermanshah faces several social, economic and environmental problems, thus confirming the need for this research. This study was completed using multi-criteria decision making (MCDM) methods, including SAW, ELECTRE and TOPSIS. The results of these three methods indicated that different urban areas in Kermanshah city have different sustainability levels and among the six urban areas, area 4 was designated as first priority. In addition, this paper offers some necessary strategies on the issues relating to the planning and management of Kermanshah city. Furthermore, the results of three methods were compared with each other. The findings of the Friedman Test showed that there is no meaningful difference between the applied methods.
Keywords: *MCDM, sustainable development, SAW, ELECTRE, TOPSIS*

Introduction

Presently, over 50% of the world’s population lives in cities. Cities form the core of social, economic, and environmental development and are also the most probable places to suffer setbacks (Connelly, 2007; Oliver, 2008; Varol et al., 2010). The statistics on the urban growth rate in the world show a significant increase in the rate of 3% in 1800 to 50% in 2008. On this basis, it is expected to increase up to 60% and 70% by 2030 and 2050, respectively (Khazaei et al., 2013). These changes have an impact on economic, social, and environmental conditions and can create problems such as social injustice, inappropriate population settlements, and climate change (Rasoolimanesh et al., 2011; Hassan et al., 2015; Huang et al., 2015). In order to face the aforementioned changes, new approaches and solutions, such as sustainable development, environmental justice, modern city lifestyle, and recently, smart development have been introduced (Taghvaei, 2013; Haikio, 2014). Sustainability was introduced for the first time in 1972 at The United Nations

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Conference on the Human Environment held in Stockholm. Also, the Agenda 21 was approved in 1992 among The United Nations Conference on Environment and Development in Rio de Janeiro (Whitehead, 2003). The concept of sustainable development was advanced to solve economic, social, and environmental problems; it is one of the most important debates worldwide, one that sees collaboration from international environmental organizations as well as the United Nations (Rotmansa et al., 2000; Rasoolimanesh et al., 2011; Maleki, 2013). Today, the proposed practical plan considers 21 fundamentals that provide the three necessities of our time, the environmental preservation of water, soil and biodiversity, which our lives are dependent upon (Willis, 2006). There are many definitions of sustainable development. Sustainable development is ‘a kind of development by which today’s generation can achieve their basic needs without limiting the future generation’s resources’. This cannot be achieved till all the factors and criteria have been defined and designed in detail (Leghaee, 1999; Berke, 2000; Jansen, 2003).

Also, the different aspects of sustainable development including, economic, social and environmental should be considered. If any aspects of sustainable development are weak, the system will be unsustainable. Urban sustainable development has the ability to develop the cities and provide the urban future generations and community's needs (Hall, 1993).

Since urban sustainable development comes from the knowledge of the conditions in urban areas, the study and review of the status of such areas is essential. In earlier research, the sustainable development level of Weifang city, of Shandong province, was studied by the AHP method. The result of this study showed that the Weifang sustainability index has been increasing in the last few years due to the environmental infrastructure improvement (Wang et al., 2012). The development of urbanization under the ecological environment restriction in the western region was done by Duan (2012). This study presents the corresponding solutions to increase the urban sustainable development quality. Different methods of multi-criteria decision making (MCDM) systems, such as SAWM, ELECTRE, TOPSIS and PROMETHEE, were used while studying the sustainability of different provinces in India (Sen et al., 2014). Evaluation of urban sustainability in 287 cities in China using TOPSIS-Entropy method has been done. Since, the level of urban sustainability in China was not high and much difference between cities exist, some strategies at urban and regional scale have been proposed (Ding et al., 2016). In another study performed by Zarrabi (2014), the social sustainability of Tehran city in Iran was assessed using factor analysis, and the level of sustainability in each urban area was defined. The same research had been done in Ilam, Iran (2009), Ahwaz, Iran (2013) by Maleki, and Orumieh, Iran (2013) by Mobaraki. Several other studies have also been conducted using MCDM methods (Viteikiene et al., 2007; Rajak et al., 2015; Zhang et al., 2016; Mokhtari Malek-Abadi et al., 2016; Liang et al., 2016; Hsueh et al., 2017).

These studies have been done by various methods and various indicators at different cities with different problems and different conditions. They indicate that there are many differences between areas in a city in terms of social, economic and environmental sustainability indicators and high inequality exist among different urban areas. According to the literatures, the cities have a trouble with legal authority and financial resources to deal with urban problems. These studies have also provided useful information on increasing the sustainable development level of cities and agreed that the urban development should be done by sustainable plans and good management and three

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important objectives should be considered including, social equality, economic development and environmental protection. While, community participation is a key role in promoting quality of urban sustainable environment.

As a country, Iran has consistently struggled with economic, social and environmental issues. Therefore, paying attention to sustainable development is essential for the nation's present and future. Several cities in Iran have developed in unsuitable ways, leading to different issues like pollution and chaos. This is particularly challenging on metropolises, which are usually focal points of population, trade, economy, culture, as well as pollution. Despite being one of the most important cities in western Iran, Kermanshah is highly unsustainable due to rapid population growth, high migration rate and increase in trade and economic activities, all of which lead to different problems. Even with the appropriate facilities, services are unevenly distributed among different parts of the city. This study aims to assess the sustainability of urban areas in Kermanshah by using the SAW, ELECTRE, and TOPSIS methods, as well as explore necessary policies to promote the sustainability level in the different areas.

Materials and methods

The study area

Kermanshah is located between latitudes 33°N and 35°N and longitudes 45°E and 47°E. The city, comprising 1.5% of Iran's total area, is located in the west and has a shared boundary with Iraq. Kermanshah Province is limited by Kurdistan in the north, Lorestan and Ilam provinces in the south, Hamedan in the east, to the country of Iraq in the west. The city of Kermanshah is the capital of the province, with a population of 818,719 people within the area of 9564 km^2 (Statistical Center of Iran, 2011). According to the population and housing census from 1966 to 2011, the population of Kermanshah city has increased from 192,072 people in 1966 to 850700 in 2011, with a positive population growth rate of 3.36%. Despite fluctuations, the city's population has always exhibited a positive growth trend (Zinatizadeh, 2013; Shirazi, 2013). Kermanshah has six urban areas, which are defined in *Table 1* and *Figure 1*.

Table 1. *Specification of each area of Kermanshah city (Statistical Center of Iran, 2015)*

Urban areas	Population	Area (Hectare)
Area 1	80689	1442
Area 2	100900	1135
Area 3	164254	1654
Area 4	151376	1469
Area 5	177408	1711
Area 6	144092	2153

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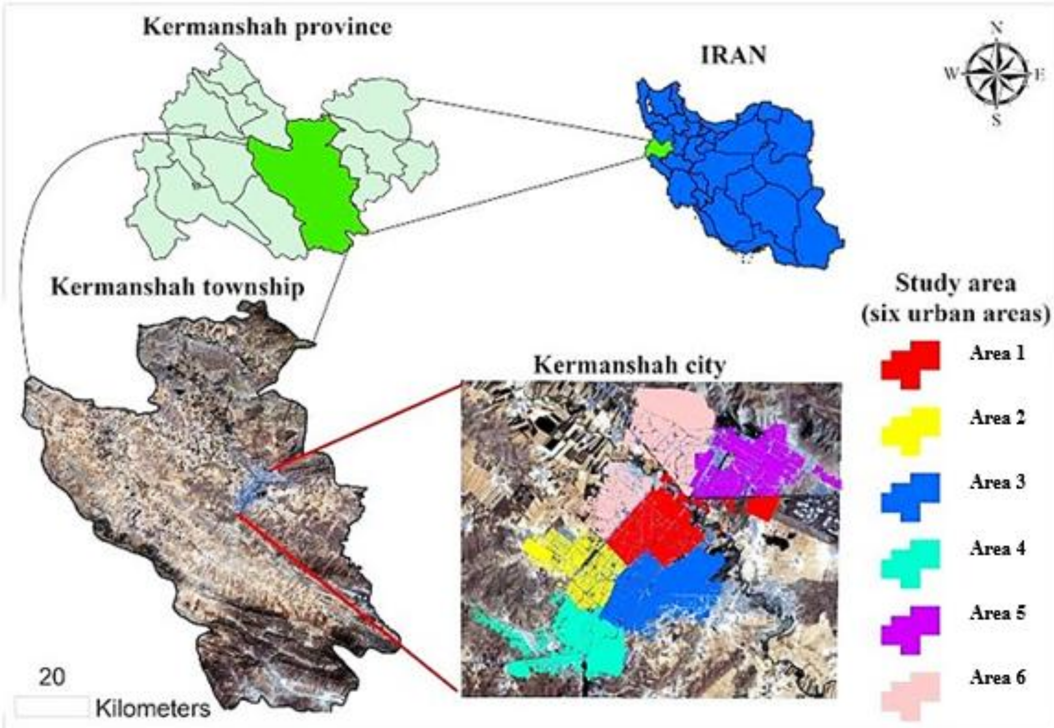

Figure 1. *Location of the study area*

In order to investigate sustainability of six urban areas in Kermanshah, a list of 44 indicators was prepared. The selection of indicators was based on expert opinions, conditions in Kermanshah city, access to the required data, and literature review (Mousavi, 2005; Zakerian, 2010; Jafaei, 2011; Aboubakri, 2012; Bahari, 2012; Raz-Dasht, 2012; Azani, 2013; Saraei, 2013; Saeedi Mafar, 2013; Akbari Nasab, 2014; Mokhtari, 2014; Li-Yin, 2011; Singh, 2009) (*Table 2*). The required data were gathered from different centres viz. province hall, municipality, department of environment, municipal water and wastewater company, waste management organizations, traffic and transportation organizations, renovation and reconstruction organizations, and statistics and information technology center. Additionally, Shannon's entropy method (Shannon, 1948) was used to determine the weight of indicators (*Table 2*).

Table 2. *List of the indicators studied and their weights in Kermanshah city (Social and welfare (1-23) Economic growth (24-31) Environmental protection (32-44))*

Indicator	Weight
(1) Population density	0.002006
(2) Literacy rate	0.000917
(3) Family size	0.008309
(4) Number of Fire station per 1000 people	0.011512
(5) Number of gas station per 1000 people	0.031225
(6) Number of toilets per 1000 people	0.023562

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(7) Number of public parking per 1000 people	0.115307
(8) Number of hotel per 1000 people	0.109761
(9) Number of not-level intersection per 1000 people	0.037687
(10) Number of central post office per 1000 people	0.007423
(11) Number of park per 1000 people	0.014057
(12) Number of police centers per 1000 people	0.028361
(13) Number of intelligent intersection per 1000 people	0.148629
(14) Number of pedestrian bridge per 1000 people	0.031043
(15) Number of Database Disaster Management per 1000 people	0.079185
(16) Number of hospital per 1000 people	0.095756
(17) Number of clinic per 1000 people	0.084947
(18) Number of drugstore per 1000 people	0.024643
(19) Numb9er of university per 1000 people	0.0869
(20) Number of sport centers per 1000 people	0.001686
(21) Number of cultural centers per 1000 people	0.009692
(22) Number of religious places per 1000 people	0.028122
(23) Number of schools per 1000 people	0.01927
(24)Unemployment rate	0.199759
(25) Employed population to 15-year-old population and more	0.208196
(26) Sponsorship rate	0.021259
(27) Number of civil projects per 1000 people	0.017955
(28) Number of shopping centers per 1000 people	0.093252
(29) Number of markets per 1000 people	0.032902
(30) Number banks per 1000 people	0.221543
(31) Number of recreation and tourism centers per 1000 people	0.205136
(32) Percent of Accidents and breakdowns in water and sewage networks	0.021924
(33) Percent of wastewater treated	0.10955
(34) Percent of Population Water network coverage	0.000461
(35) Percent of Population network wastewater coverage	0.000921
(36) Percent of source separation of solid waste	0.216273
(37)Waste production per capita	0.0043
(38) Percent of Semi-mechanized collection of household waste	0.004441
(39) Number of industrial centers per 1000 people	0.191567
(40) Number of database associated with environment per 1000 people	0.14267
(41) Green space per capita	0.111328
(42) Number of traffic jams per 1000 people	0.053538
(43) Percent of travel at peak hours	0.045457
(44) Percent of old area	0.09757

Source: (Mousavi 2005, Zakerian 2010, Jafaei 2011, Aboubakri 2012, Bahari 2012, Raz-Dasht 2012, Azani 2013, Saraei 2013, Saeedi Mafar 2013, Akbari Nasab 2014, Mokhtari 2014, Li-Yin 2011 and Singh 2009)

Following this, the MCDM methods were used for prioritizing the urban areas. The MCDM method is a branch of operations research models which cope with decision problems that have a number of criteria (Pohekar et al., 2004). In this study, one method from each subgroup of the MCDM methods was chosen. The selective methods include Elimination and Choice Expressing Reality (ELECTRE) from the concordance sub-group, the Technique for Order Preference by Similarity to Ideal Solutions (TOPSIS) from the compromising sub-group, and Simple Additive Weighting (SAW) from the scoring sub-group of the MCDM methods. These methods are further described in the following sections.

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SAW

The simple additive weighting (SAW) is one of the simplest methods of the MCDM methods (Churchman et al., 1954):

Normalize the decision matrix (N):

According to the *Eq. 1*, the decision matrix is normalized using of linear method.

$$n_{ij} = \frac{a_{ij}}{Max\ aij} \quad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 1)}$$

Calculate the weighted normalized decision matrix (V):

The weighted normalized value (v_{ij}) is obtained according to *Eq. 2*.

$$V_{ij} = N_{ij}W_j \quad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 2)}$$

Where w_j is the weight of the *i*th indicator.

Select the alternative with the highest overall performance value (A):*

$$A^* = \{A_i Max \sum n_{ij} w_j\} \qquad \text{(Eq. 3)}$$

ELECTRE

ELECTRE was introduced at the end of 1980th (Roy 1968). ELECTRE Is an outranking method based on outranking relation and concordance analysis. So, this method does not necessarily results in ranking the alternatives (Velasquez et al 2013; Taha et al 2013).

Normalize the decision matrix (N):

The normalized value (n_{ij}) is obtained according to the *Eq. 4*.

$$n_{ij} = \frac{x_{ij}}{\left[\sum_{i=1}^{m} x_{ij}^2\right]^{\frac{1}{2}}} \quad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 4)}$$

Calculate the weighted normalized decision matrix (V):

The weighted normalized value (v_{ij}) is obtained according to *Eq. 5*.

$$V_{ij} = N_{ij}W_j \quad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 5)}$$

Where w_j is the weight of the *i*th indicator.

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Formation the concordance matrix set

$$S_{k,l} = \{j \mid Vkj \geq Vij\}\ , \quad j = 1, \ldots, m$$

$$S_{k,l} = \{j \mid Vkj \leq Vij\}\ , \quad j = 1, \ldots, m$$

Formation the discordance matrix set

$$D_{k,l} = \{j \mid Vkj < Vij\}, \quad j = 1, \ldots, m$$

$$D_{k,l} = \{j \mid Vkj > Vij\}, \quad j = 1, \ldots, m$$

Determine of concordance matrix (I_{kl})

In this stage, the concordance matrix is calculated using *Eq. 6*. This matrix is an m*m matrix, which diameter of that, does not have any element. The other elements (entries) of this matrix can be calculated via summation of indicator weights that belong to concordance group.

$$I_{kl} = \sum w_j\ ,\quad j \in A_{k,l} \qquad \text{(Eq. 6)}$$

Determine of discordance matrix (NI):

In this stage, the discordance matrix is calculated using *Eq. 7*. This matrix is an m*m matrix. The diameter of this matrix does not have any elements and other elements can be calculated from weighted normalized matrix according to *Eq. 7*.

$$NI_{kl} = \frac{Max\left|V_{ij} - V_{lj}\right|, j \in D_{k,l}}{Max\left|V_{ij} - V_{lj}\right|, j \in all\ indicators} \qquad \text{(Eq. 7)}$$

Calculate the effective concordance matrix (H):

To create this matrix, first we need to determine a threshold. If each element of matrix I, be bigger or equal to that, that factor in matrix H, will be equal one, otherwise 0. From the *Eq. 8*, the threshold for the matrix is calculated.

$$I = \sum_{i=1}^{m} \sum_{k=1}^{m} I_{k_l} / m(m-1) \qquad \text{(Eq. 8)}$$

If, $I_{ki} \geq \bar{I}$, $H_{ki} = 1$ *and* *If,* $I_{ki} < \bar{I}$, $H_{ki} = 0$

Calculate the effective discordance matrix (G):

In this stage, the effective discordance matrix can be calculated based on the *Eq. 9*

$$\overline{NI} = \sum_{i=1}^{m} \sum_{k=1}^{m} NI_{k_l} / m(m-1) \qquad \text{(Eq. 9)}$$

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$$If, \quad NI_{ki} \geq \overline{NI}, \quad G_{ki} = 0 \qquad and \qquad If, \quad NI_{ki} < \overline{NI}, \quad G_{ki} = 1$$

Determine the effective final matrix (F):

The effective final matrix can be calculated by combination of effective concordance and effective discordance matrix based on the *Eq. 10*.

$$F_{kl} = H_{kl} G_{kl} \qquad \text{(Eq. 10)}$$

TOPSIS

TOPSIS was proposed by Hwang and Yoon in 1981. The basic concept of this method is that, the chosen alternative should have the closest distance from the ideal solution (Velasquez et al., 2013; Taha et al., 2013).

Normalize the decision matrix (N):

The normalized value (n_{ij}) is obtained according to *Eq. 11*.

$$n_{ij} = \frac{x_{ij}}{\left[\sum_{i=1}^{m} {x_{ij}}^2\right]^{\frac{1}{2}}} \qquad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 11)}$$

Calculate the weighted normalized decision matrix (V):

The weighted normalized value (v_{ij}) is obtained according to *Eq. 12*.

$$V_{ij} = N_{ij} W_j \qquad i = 1, \ldots, m, \quad j = 1, \ldots, n \qquad \text{(Eq. 12)}$$

Where w_j is the weight of the *i*th indicator.

Determine of positive ideal solution and negative ideal solution

Positive ideal solution (V_j+) =vector of the best values of each indicator (*V*)
Negative ideal solution (V_j^-) = vector of the worst values of each indicator (*V*)
The best values for the positive indicators are the biggest and for the negative indicators are lowest values.

Determine of Euclidean distance to positive and negative ideals:

Euclidean distance of each alternative from positive ideal (d_j^+) and negative ideal (d_j^-) can be calculated based on *Eq. 13* and *14*.

$$d_i^+ = \sqrt{\sum_{j=1}^{n} \left(V_{ij} - V_j^+\right)^2} \quad , \ i = 1,2, \ldots, m \qquad \text{(Eq. 13)}$$

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$$d_i^- = \sqrt{\sum_{j=1}^{n}\left(V_{ij} - V_j^-\right)^2} \quad , \ i = 1,2,\ldots,m \qquad \text{(Eq. 14)}$$

Determine the relative closeness to ideal solution (CL):*

Determination of relative closeness of each alternative to an ideal solution can be calculated based on *Eq. 15*.

$$CL_i^* = \frac{d_i^-}{d_i^- + d_i^+} \quad , \quad i = 1,2 \ldots,n, \quad 0 \leq CL_i^* \leq 1 \qquad \text{(Eq. 15)}$$

Ranking the preference order

Alternatives having bigger CL, are better. According to relative closeness of each alternative to an ideal solution, the ranking order of alternatives can be determined and the best alternative is selected.

Results

Based on the data obtained, the sustainability levels of six urban areas were evaluated using three methods under the MCDM, and three aspects of sustainability, including social, economic and environmental.

Urban areas ranking by SAW

The results obtained from SAW in three categories (social, economic, and environmental) are shown in *Table 3*. The best alternative selected is the one where the sum of the weighted normalized values is greater than other values.

Table 3. *Results obtained from SAW method*

Area	Sum of environmental indicators values	Rank	Sum of economic indicators values	Rank	Sum of social indicators values	Rank	Sum of indicators values	Rank
1	0.764	1	0.817	1	0.471	4	2.052	4
2	0.362	5	0.695	2	0.489	3	1.555	3
3	0.538	3	0.547	4	0.626	1	1.711	1
4	0.602	2	0.560	3	0.600	2	1.762	2
5	0.330	6	0	5	0.290	6	0.62	6
6	0.368	4	0	5	0.392	5	0.76	5

According to the results, Area 1, with a value of 2.052, is more sustainable than the others. Three areas (4, 3, and 2) are ranked in the second, third, and fourth place, with

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values of 1.762, 1.711 and 1.555, respectively. Areas 6 and 5 are graded as fifth and sixth, with 0.76 and 0.62, respectively.

Urban areas ranking by ELECTRE

The results of urban area ranking in three categories (social, economic, and environmental) using the ELECTRE method are shown in *Tables 4, 5, and 6*.

Table 4. *The final dominance matrix of social indicators*

Final dominancy	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6
Area 1	-	0	0	0	1	1
Area 2	0	-	0	0	1	1
Area 3	0	1	-	0	1	1
Area 4	0	1	0	-	1	1
Area 5	0	0	.	0	-	0
Area 6	0	0	0	0	1	-

Table 5. *The final dominance matrix of economic indicators*

Final dominancy	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6
Area 1	-	1	0	0	0	1
Area 2	0	-	0	0	0	1
Area 3	0	0	-	0	0	1
Area 4	1	1	1	-	1	1
Area 5	0	1	0	0	-	1
Area 6	0	0	0	0	0	-

Table 6. *The final dominance matrix of environmental indicators*

Final dominancy	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6
Area 1	-	1	1	0	1	1
Area 2	0	-	0	0	0	0
Area 3	0	0	-	0	1	0
Area 4	0	1	0	-	1	1
Area 5	0	0	0	0	-	0
Area 6	0	0	0	0	0	-

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Based on the results, two areas (4 and 1) have the maximum dominance, followed by areas 3, 2, 5, and 6, in that order.

Urban areas ranking by TOPSIS

For the current study, the relative closeness of each alternative to the ideal solution, in three categories (social, economic, and environmental), was calculated (*Table 7*).

Table 7. *Relative closeness of each alternative to an ideal solution*

Alternatives	Social indicators	Economic indicators	Environmental indicators	Overall closeness to ideal solution
Area 1	0.437	0.52	0.627	1.594
Area 2	0.437	0.349	0.207	1.083
Area 3	0.575	0.578	0.449	1.602
Area 4	0.537	0.913	0.579	2.029
Area 5	0.197	0.557	0.21	0.964
Area 6	0.319	0.196	0.361	0.876

According to the results obtained by this approach, Area 4, with a score of 2.029, is in better condition when compared to the others. Three urban areas (3, 1, and 2) are ranked second, third and fourth, with scores of 1.602, 1.594 and 1.083, respectively. The remaining areas (5 and 6), with scores of 0.964 and 0.876 scores, are ranked fifth and sixth grade, respectively.

Final ranking of urban areas

The final ranking result is based on the average of the ranks obtained from different methods (Momeni 2013). Accordingly, the final rankings of the six urban areas are shown in *Table 8*.

Table 8. *Final ranking results*

Area	MCDM methods			Averaged rank
	SAW	ELECTRE	TOPSIS	
Area 1	1	2	3	2
Area 2	4	4	4	4
Area 3	3	3	2	2.6
Area 4	2	1	1	1.3
Area 5	6	5	5	5.3
Area 6	5	6	6	5.6
Area 4 > area 1 > area 3 > area 2 > area 5 > area 6				

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Discussion

Based on this study, it is evident that different urban areas of Kermanshah city have different sustainability levels. Area 4 is listed as the first priority, followed by areas 1, 3, 2, 5 and 6, respectively. Due to the centrality of area 4, more social and public services, including health, culture, education, entertainment, sports, as well as city infrastructure and administrative buildings are located here. Area 1, which is predominantly occupied by the wealthy, is characterized by high economy; collaboration on environmental projects receives particular attention in this area (due to the location of the administrative section), and it is therefore more sustainable. In comparison, in two areas (2 and 3), the disorganized structure of streets and alleys leads to traffic problems, water and sewage-network-related accidents and difficulties in municipal waste management. Furthermore, Areas 5 and 6 are not well-developed due to rural migration, cheap land price and semi-rural structure. Overall, the main factors contributing to unsustainable conditions in six urban areas of Kermanshah are high unemployment rate, high solid waste production, unequal distribution of city services and infrastructures, the old structure of the city, inefficient public transportation system, lack of attention to renovation projects in older areas and social discrepancies.

Based on these limited factors, particular strategies need to be considered. These include the provision of basic services, decentralization, promoting public participation in urban planning, infrastructure establishment for proper development, building recreation and tourism centers in the city, enhancing relationship with Iraq, improving economic prosperity and reducing marginal jobs. It is further important to draw the attention of local, regional, and national planners towards creating a special economic zone in Kermanshah province, which can create various employment opportunities, and assist in the development of waste management programs and wastewater treatment plants, as well as improve public transportation, and increase green spaces across the city.

In the present study, the results from SAW, ELECTRE, and TOPSIS methods were compared using the Friedman test. Based on the sig (0/93) value, no meaningful difference was found among the methods. As seen in *Table 8*, areas 5 and 6, with the lowest sustainability levels are ranked similarly in all three methods. Areas 1, 3, and 4, with comparably superior sustainability levels were ranked high in all methods, while area 2 in ranked fourth in all of the methods. In the other words, all three applied methods have resulted in similar rankings.

It is probable that due to the use of a single method for weighting (Shannon’s entropy), the results achieved from all three methods were similar. This suggests that in the process of alternatives prioritization, the weight of indicators is more important than the method being used (Janic et al., 2002; Zareie et al., 2011). On the other hand, the three methods applied in this study belong to compensatory MCDM methods. These refer to methods where trade-offs between the indicators are allowed (XU et al., 2001); that is, a decline in one indicator's attributes is acceptable if it is compensated by an increase in another indicator's attribute. Thus, one of the characteristics of compensatory methods is the closeness among the rankings (Ebrahimi et al., 2014). Many previous studies have shown similar results in final rankings with the use of different MCDM methods, which corresponds to the current study (Chu et al., 2007; Hoshyar et al., 2011; Momeni et al., 2011; Shirouyehzad et al., 2011; Bordbar, 2013).

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Despite the similarity between the results obtained from the three different methods, the authors propose TOPSIS as the most suitable and practical method. This selection is based on the advantages and characteristics of this method as compared to the rest. The benefits of TOPSIS are listed below - (Srdjevici, 2004; Falsoleiman et al., 2013; Kolios et al., 2016):

- It is capable of merging several quality and quantity indicators simultaneously.
- The method is characterized by simplicity and high speed.
- The system function is desirable and acceptable.
- The desirability of applied indicators in solving a problem can be steadily increasing (or decreasing).
- The method allows changing the primary data, and subsequently, changing the functions and outcome.
- Prioritizing in this method is done based on similarity to the ideal solution, such that the final option will be close to the ideal answer and far from the wrong answer.
- In case some of the indicators are not desirable and need to be decreased, or some others are desirable and can be increased, TOPSIS can easily calculate the ideal solution through a combination of the best values obtained from all criteria.
- TOPSIS considers the best and the worst answers simultaneously, based on closeness to the ideal solution.
- The outcomes can express the priorities quantitatively.
- *Table 9* compares the specifications for the different methods.

Table 9. *Comparison of characteristics of methods (SAW, ELECTRE, and TOPSIS)*

No	Feature	TOPSIS	SAW	ELECTRE
1	Compared data type	Quality and quantity	Quality and quantity	Quality and quantity
2	Stability of the results	+	+	+
3	Simplicity	*	***	**
4	Intelligibility	*	*	**
5	Creditability	**	*	**
6	Flexibility	**	*	**
7	Applicability	*	*	**
8	Provide better results	*	*	**
9	Calculation precision	*	*	**
10	Providing details	*	*	**
11	Regarding the decision-maker	*	*	*
12	Sensibility to the weighting	***	*	*
13	Ability in pair comparison	+	-	-
14	The ability to analyze a large number of data	+	+	+
15	Ability to manage low quality input data	+	-	-
16	Computing speed	*	***	**

*(+ = have, - = does not have, * = low, ** = medium, *** = high)*
Source :(Duckstein et al., 1982; Goicoechea et al., 1982; Hobbs, 1986; Hobbs 1992; Srdjevici, 2004; Cavallaro et al., 2005; Caterino et al., 2008; Nikolic et al., 2009; Azar et al., 2010; Achilas et al., 2011; Aruldoss et al., 2013; Amoushahi et al., 2015; Falsoleiman et al., 2013; Hatami Marbini et a. 2013; Herva et al. 2013; Mohammadi Zanjirani et al., 2013)

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Conclusion

Multi-criteria decision making methods were applied to assess the sustainability of urban areas in Kermanshah city. In this research, the authors applied the ELECTRE, TOPSIS and SAW methods for the evaluation of sustainability of urban areas using 44 indicators at three social, economic and environmental aspects. In addition, The Friedman test showed there is no significant difference between these MCDM methods. The results showed that among the six urban areas, areas 4, 1 and 3 are more sustainable than the other areas. Totally, Kermanshah city is not at good condition and is far from sustainability. Therefore, in order to achieve sustainability, especially in more deprived areas, some strategies should be done such as creating jobs, more attention to economic in undeveloped areas, cultural solution and collaboration of people in environmental issues. Equal distribution and decentralization of services and allocating funds to poor areas must be considered. The study also pointed out the advantages and disadvantages related to the application of the selected methods and the TOPSIS method was preferred due to its advantages over the other methods.

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ISOTOPIC (δ^{13}C AND δ^{15}N) VARIATIONS IN TROPICAL RIVER SEDIMENTS OF KELANTAN, MALAYSIA: A RECONNAISSANCE STUDY OF LAND USE IMPACT TO THE WATERSHED

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Abstract. Intensification of sedimentation process has resulted in shallower river, thus increasing their vulnerability to natural hazards (i.e. climate change, floods). Considering the impact of soil erosion to the sedimentation, identification of the main source of erosion is critical to watershed management. Stable isotope of carbon (δ^{13}C) and nitrogen (^{15}N) were carried out in Kelantan catchment. Results from δ^{13}C showed that C_3 type plant represents as major plant (-29.10‰ to -23.60‰) in the river. While for δ^{15}N, the isotopic variations demonstrate distinct pattern of dry (Southwest Monsoon, SWM) and wet (Northeast Monsoon, NEM) seasons, suggesting significant pollutants washout from terrestrial (due to agricultural activities) to the water bodies. In addition, the Kelantan river system is an autochthonous as determined by carbon to nitrogen (C:N) ratio. Essentially, this study will serve as a precursor of future study to understand the impact of anthropogenic activities on carbon and nitrogen cycles in tropical catchment.
Keywords: *erosion, sedimentation, carbon, nitrogen, cycle, tropical, deforestation, stable isotopes, agriculture, land use, C_3 plants, C:N ratio, water quality, sediment yield*

Introduction

Kelantan basin is located in northeast of Peninsular Malaysia. The main river is known as Kelantan river with the total length of 248 km, with draining that flows northwards in the direction of the South China Sea. The catchment area is approximately 11,900 km^2 (Yen and Rohasliney, 2013) occupying more than 85% of Kelantan state. Along the river, the topography is comprised of rain forested hills, lowland forests and limestone caves. Currently, the type of activities that occur around the area include deforestation, vegetation and urbanization (Basarudin et al., 2014).

Land use activities that degrade natural landscape through clearing of tropical rainforests, unsustainable agriculture and rapid urban expansion have had significant impacts to hydrological conditions and ecological processes (Adnan et al., 2014; Hadi et

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al., 2017) in the Kelantan watershed. It is also vulnerable to climate change (Huang and Lo, 2015; Zafirah et al, 2017). Changes in geographical area leads to alteration of hydrological process. Impact of various land use activities include flowing of fine sediment into the river, due to soil surface erosion (Chen et al., 2015). Soil erosion is the result of overland flow as rainfall occurs, which in turn results in serious environmental problem if not properly controlled (Mahabaleshwara and Nagabhushan, 2014). Increasing sedimentation in the river can cause extensive catchment flooding due to overflow at the river, water pollution and poor drainage system (Kithiia, 2012; Zafirah et al., 2017). Mitigation process is often delayed due to lack of capability to identify the cause and source of erosion. This paper will discuss the application of environmental isotopes technique to identify the source of erosion in Kelantan catchment. Sediments in the Kelantan rivers were collected and analyzed by using Isotopic Ratio Mass Spectrometer (IRMS) to measure the δ^{13}C and δ^{15}N and Perkin Elmer 2400 Series II CHN Elemental Analyzer for C:N analysis in the sample.

Soil erosion and sedimentation mechanism

Soil erosion requires three steps to trigger the process: (i) detachment, (ii) transport and (iii) deposition (Pidwirny, 2008; Zafirah et al., 2017). Detachment process is defined as a particle that is disengaged from the main subject. It requires the procedure of breaking the bond that binds the particle together. Factors of weathering in physical, chemical and biological state causes the bond particle in the subject to weaken and detach.

It is then followed by transportation process, whereby the particle moves with the presence of the medium or agent at high velocity. This step depends on mass of particle, size, shape, surface configuration and medium type to undergo the process (Pidwirny, 2008; Safaei et al., 2014). Finally, it ends with deposition process, whereby the velocity of the medium reduces or increases in particle resistance (Stephen, 2016).

Sedimentation is a result of erosion process where it refers to the accumulation of particle that is carried out by a medium such as water, wind or ice for transportation. Sedimentation process is a natural process, although it may be influenced by anthropogenic factors. Transported sediments can be chemical, organic material or inorganic, mineral matter and pollutants. Increase in sediments and siltation have threatened the surrounding ecology, irrigation of river and aquatic habitat (Huggins et. al., 2007; Kjelland et al., 2015). It also makes the river to become more shallow and easier to overflow.

Stable isotope as tool in fingerprint detection

Stable isotope analysis is one of the potential tool to detect and trace the fingerprint of organic matter in the environment (Liu et al., 2017) such as aquatic, water and sediment, terrestrial, soil, plant and others. The stable isotope carbon and nitrogen were used to analyze the values, therefore determining the categories and understanding the process of sedimentation in the catchment (Tue et al., 2011). Carbon isotope were used to differentiate the type of plants present in the selected area. δ^{13}C have been used to determine the C_3 and C_4 plants, which indicates the photosynthesis process that plants undergo (Boullion et al., 2003a; Tue et al., 2011).

The ranges of C_3 plants are from -32 to -22‰ and -16 to -9 for C_4 (Medina et al., 1999; Kendall et al., 2001; Schaal et al., 2008; Kohn, 2010), hence it is useful as an

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ecosystem components tracer (Bouillon and Boschker 2006; Werner and Máguas, 2010; Gilbert et al., 2012). Variations of δ^{13}C provide clues for further investigation on environmental condition and land use activities in the watershed (Cravotta, 2001; Fushan et al., 2014).

Stable nitrogen isotope is commonly used to detect the sources of pollution, and it is made up of several of classes such as soil organics, fertilizers, animal or sewage waste and precipitation (Heaton, 1986; Lim et al., 2010; Zhao et al., 2016). Different of δ^{15}N is determined by fractionation process, which consist of four types, including equilibrium, kinetic, mass independent or transient kinetic isotope fractionation (Heaton, 1986; Dähnke and Thamdrup, 2013; Ryabenko, 2013). From different values, it will then be used to classify the possible sources based on the fingerprint, as well as the activities nearby the sampling location. Therefore, this approach is used to identify the possible sources of sedimentation in Kelantan catchments.

Material and Methods

Study area

Malaysia is located near the equator and is one of the tropical countries that is hot and humid all year round. The average temperature is about 27^{0}C and receives rainfall on average 2500 mm annually. Peninsular climate differs from Malaysian Borneo as the peninsular is directly affected by wind and is exposed to the El Niño effect. The phenomena cause dry season as it reduces the amount of rainfall. Climate change has a significant impact on Malaysia as it is crucial in determining the sea level as well as amount of precipitation, which results either in drought or extensive rainfall, which increases the flood risks. In addition, Malaysia also experiences monsoon seasons, Northeast Monsoon (NEM) from November to March and Southeast Monsoon (SEM) from May to September. Monsoon occurs due to atmospheric pressure patterns in Southeast Asia that results from different pressure between Asia continent and land mass in Australia, known as Inter-Tropical Convergence Zone (ITCZ). NEM hit Peninsular Malaysia during northern hemisphere during winter seasons, together with high pressure from China and low pressure in Australia thus combination of forces the ITCZ in south areas. Opposite circulation occurs when northern hemisphere experience summer seasons, low pressure in Asia and high pressure in Australia forcing the ITCZ in northwards resulting SWM in Peninsular Malaysia. Both seasons, NEM and SWM play the main factors in determining the amount of precipitation in Malaysia (Loo et al., 2015). However, between these two monsoons, NEM give extra rainfall events compared to SWM (Suhaila et al., 2010).

This study focuses in Kelantan catchment area, located at east coast of Peninsular Malaysia (*Figure 1*). The Peninsular Malaysia lies between 1^0 and 7^0 North and 99^0 to 105^0 East, extending 748 km SSE-NNW and 322 km ENE-WSW. The landscapes of peninsular at inland areas are mainly from denudation terrains of bedrocks, resulting from weathering and erosion process. The range of Titiwangsa (highland) located somewhat at the middle of Peninsular region, separates the eastern and western part of the region. In addition, it is composed mainly of granite with some regions of metasedimentary rocks (Ishak, 2014). These series of mountain ranges play a crucial role in creating the streams and rivers network pattern (Hutchison and Tan, 2009).

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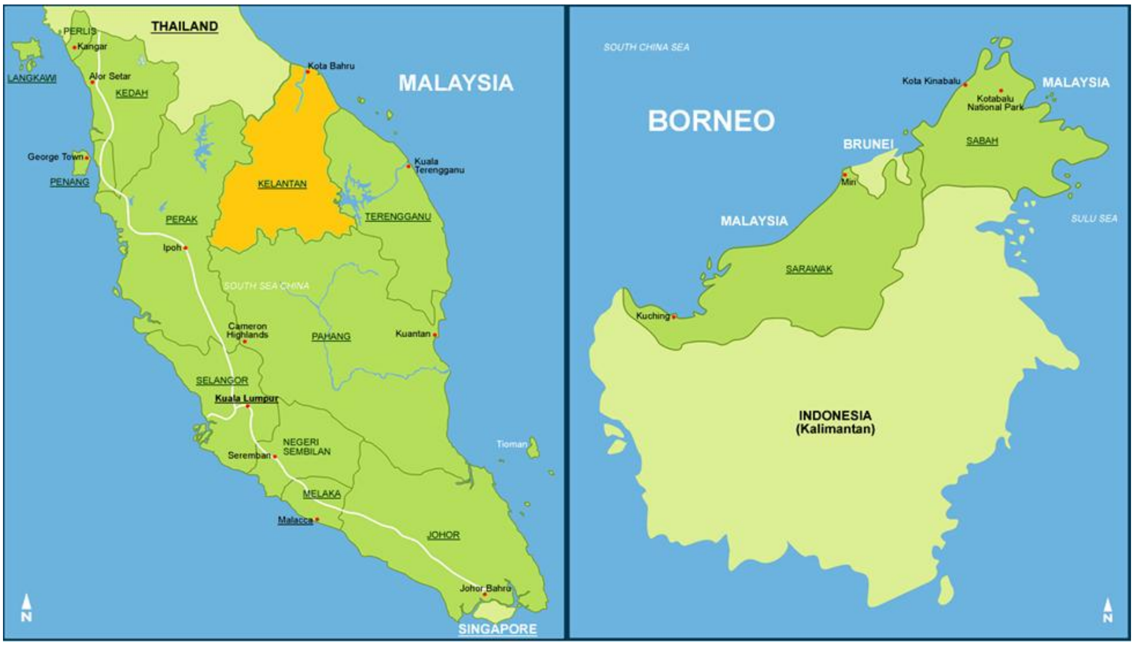

Figure 1. *Malaysia map (World Map, 2017)*

Kelantan Basin

The study area covers the catchment around Kelantan state which is located in the northeast of Peninsular Malaysia lies between 4^0 40’ and 6^0 12’ North and 101^0 20’ and 102 20’ East (*Figure 2*). Kelantan catchment comprises an area about 11,900 km², (Yen and Rohasline, 2013). Three main rivers at Gua Musang District which are Nenggiri River, Betis River and Broke River were selected. The dominant land use of this area is agriculture, native forest and countryside native. The next river is Lebir River located at Kuala Krai district. Pergau River which is located in Jeli district, has one of the highest waterfalls in Southeast Asia. The river merges with Galas River. The land use of this area comprises of croplands and new settlements. Kelantan river, which is the third longest river in Peninsular Malaysia, is also included in this study.

Climate and hydrology of Kelantan Basin

The regional climate of Kelantan state has temperatures ranging between 21 to 32 ^{o}C and recurrent rain throughout the year. However, it is exposed with extra rainfall in Northeast Monsoon from November to March every year. The average rainfall is about 2062 to 2543 mm per year and humidity is constantly high on the lowlands ranging between 82% and 86% annually (Irwan et al., 2013).

Vegetation and land use in Kelantan watershed

Majority of Kelantan’s land cover consist of forest. Its economy constitutes mainly from agriculture industries dominated by oil palm plantation, rubber, paddy and other cash crops.

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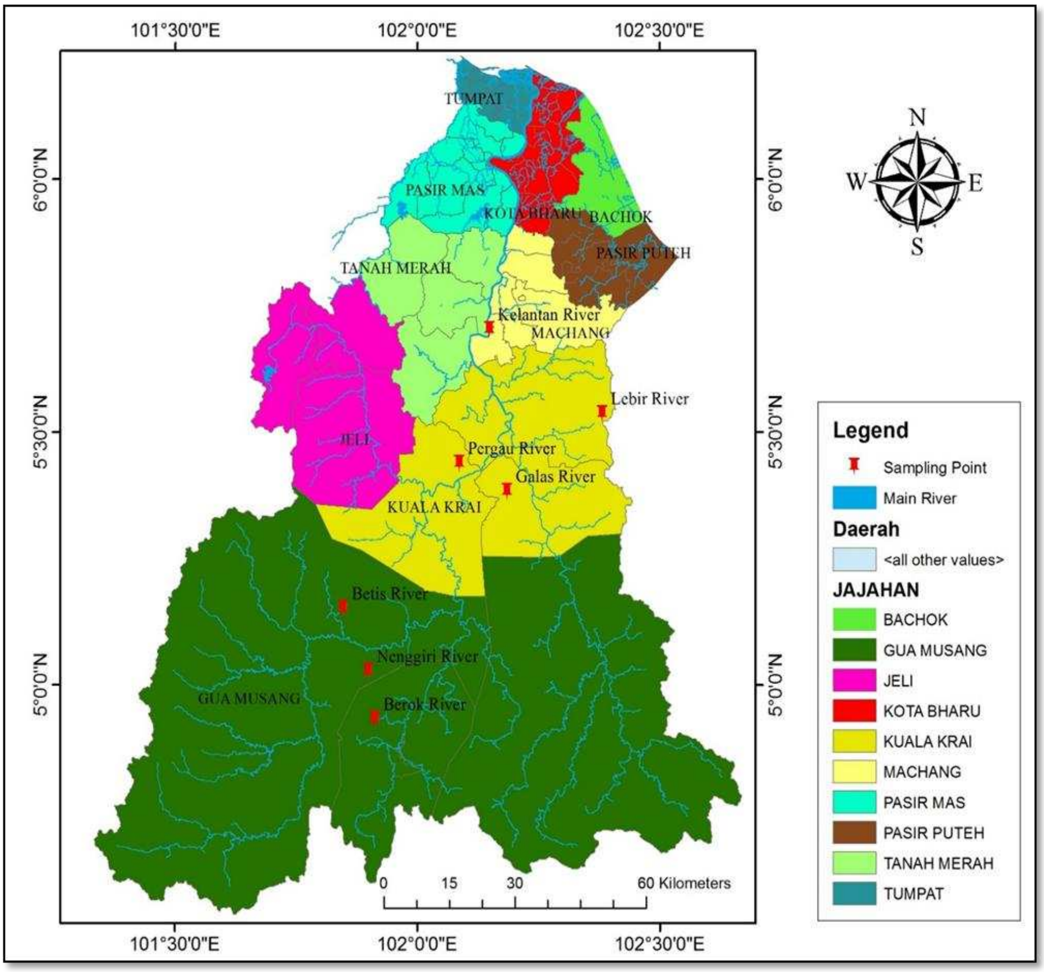

Figure 2. *Map of the Kelantan river and study sites*

Sample collection and analytical procedures

Sediment sample collection was carried out in July 2015 and January 2016. The sediments were sampled at seven selected stations; Broke, Betis, Nenggiri, Lebir, Galas, Pergau and Kelantan river. The sediment was sampled at 10" of top sediment, with approximately 100 g per sediment sample by using grab sampler and subsequently packed into zipper bag, and is preserved in a cold storage box. Samples were stored in the laboratory accordingly (Shanbehzadeh et al., 2014).

Sample preparation

In the laboratory, 50 g of sediment samples were washed by using 10% of hydrochloric acid prior to remove carbonates (Kennedy et al., 2005) and rinsed with distilled water for three times. The samples were then dried at 80 to 90 ºC to remove moisture. The subsample was then grounded into powder form. Grinding was performed using a mortar and pestle. The sample were finally sieved through a 425µm sieve and stored in glass jars.

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Stable isotope analysis

Each sediment sample was weighed into small tin capsule (8 x 5 mm) for 12mg in triplicates. The sample was folded and compressed into a tight ball before being loaded into an auto-sampler. All the replicated samples were analysed for stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition using Flash EA 2000 elemental analyser (ThermoScientific, Waltham, MA) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo, Milan, Italy).

Raw isotope ratios from the analysis were normalized to the international scales using USGS-40 and USGS-41 reference materials (~0.5 mg, respectively) assayed with the unknown samples. For quality control material, Urea (IVA-Analysentechnik GmbH & Co., Germany) was used to correct for drift. It was measured for every 12 samples with known values of $\delta^{13}C$ = -40.81‰ and $\delta^{15}N$ = -0.49‰. Variations in stable isotope ratios were reported as parts per thousand (‰) deviations from internationally accepted standards which are Vienna Pee Dee Belemnite (VPDB) for carbon, atmospheric nitrogen (AIR) for nitrogen, in the delta (δ) notation.

The δ notation is defined using the following Equation (1):

$$\delta\ (‰) = (\ R\text{sample} \ /\ R\text{standard}\ -\ 1) \qquad \text{(Eq.1)}$$

where R_{sample} is the isotope ratio ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) of the sample, and $R_{standard}$ is the isotopic ratio of the international reference materials.

Carbon Nitrogen Ratio (C:N Ratio) Analysis

Same as isotope analysis, each sediment sample was weighed into small tin capsule (8 x 5 mm) for 2 mg in triplicates. The sample was folded and compressed into a tight ball before being loaded into an auto-sampler. All the replicated samples were analyzed for carbon and nitrogen composition using Perkin Elmer 2400 Series II CHN Elemental Analyzer (Perkin Elmer).

Samples were introduced from an autosampler into a combustion furnace with a temperature of 925°C. The resulting gases (CO_2, NOx, and H_2O) were passed through combustion and reduction columns, mixed, and separated through thermal conductivity detector (TCD) gas chromatography column (Stephen et al., 2011). Acetanilide standards were run every triplicates samples to ensure proper instrument operation.

The C:N ratios is defined using the following Equation (2):

$$\frac{C}{N} ratio = (\frac{(\%C/\%N)}{(14/12)} \qquad \text{(Eq.2)}$$

Water quality and sediment yields data

Water quality data (2004 - 2014) were provided by Department of Environment (DOE). The parameters include chemical oxygen demand (COD), biochemical oxygen demand (BOD), suspended solids (SS), pH, dissolved oxygen (DO), and Ammoniacal nitrogen (NH3-N). Besides, data of sediment yields in Kelantan were also retrieved from Department of Irrigation and Drainage (DID) Kelantan. The data is a 30-year dataset that ranges from 1980 to 2009. Both secondary data were analyzed using Principal Component Analysis (PCA).

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Results and Discussion

Stable isotopes analysis of $\delta^{13}C$ and $\delta^{15}N$ in sediment at different catchment area in Kelantan

Plant types can be divided into three categories; C_3, C_4 and Crassulacean acid metabolism (CAM) based on carbon fixation of carbon dioxide during photosynthesis process. Every plant group will produce different $\delta^{13}C$ signatures of the organic carbon. C_3 plant is ranged from approximately -34‰ to -23‰, C_4 -18‰ to -12‰ and CAM -32‰ to -12‰, (Brand, 1996; Faure and Mensing, 2005). In Malaysia, there are less number of CAM plants, as it is usually found in dry or arid condition (Masrahi et al., 2012), which allows stomata to remain shut during the day.

A distinct inclination of $\delta^{13}C$ sediment values emerged in all seven studied locations were plotted in *Figure 3*. In overall, the mean value for $\delta^{13}C$ for July was -27.05 ± 1.02‰ followed by January, -26.80 ± 1.76‰ (*Table 1*). The results from both dry and wet seasons in Kelantan catchment showed that the value ranged from -29.10‰ to -23.60‰, which represent C_3 type plants suggested that terrestrial plants (allochthonous) were the main source of sediment contribution in all seven rivers.

The mean of $\delta^{13}C$ at Brok samples is -26.81 ± 1.46‰ and Betis is -24.90 ± 0.98‰ (*Table 1*). Both are located at Gua Musang district. The major type of vegetation here is rubber (Hevea brasiliensis) (Da Matta et al., 2001), tapioca (*Manihot esculenta*) (Calatayud et al., 2002) and banana (*Musa spp.*) (Janssens et al., 2009), all representing C_3 type plants. Nenggiri is the main river of Betis and Brok tributaries and average of the $\delta^{13}C$ in sediments is -26.08 ± 1.28‰ (*Table 1*). Galas and Pergau have almost the same average of $\delta^{13}C$ in sediments, -27.36 ± 0.60‰ and - 27.12 ± 0.84‰ (*Table 1*) respectively. The rivers are located in Dabong, south of Kelantan. Rubber estate appears as the main land use activity, followed by small farming of banana and tapioca.

Lebir River which is located in Kuala Krai district, with average of $\delta^{13}C$ is -27.99 ± 0.88‰ (*Table 1*), of which palm oil plantation is the main land use activity representing approximately 80% of the catchment area. According to Lamade et al., (2009), $\delta^{13}C$ of oil palm (*Elaeis guineensis*) leaves is around ≈-27‰, which is also categorized as a C_3 plant type. For Kelantan river sediments located at the downstream catchment, the mean value of $\delta^{13}C$ is - 28.22 ± 0.82‰ (*Table 1*). Small farming activities like banana, cocoa, tapioca and rubber estate are present around the catchment area.

Table 1. *Isotopic composition of stable isotope $\delta^{13}C$ in sediment*

Stable Isotope $\delta^{13}C$ [‰]								
	Rivers							Average season
Season	Broke	Betis	Nenggiri	Galas	Pergau	Lebir	Kelantan	
Jul (SEM)	-25.85	-25.71	-27.19	-28.31	-28.21	-26.48	-27.42	-27.05 ± 1.02
	-26.79	-24.15	-26.43	-27.07	-27.53	-27.51	-27.51	
	-28.07	-25.81	-27.84	-26.94	-27.79	-27.96	-27.48	
Jan (NEM)	-25.55	-23.60	-24.88	-27.75	-26.10	-28.68	-28.88	-26.80 ± 1.76
	-25.56	-25.78	-24.74	-26.65	-26.72	-28.65	-28.90	
	-29.05	-24.34	-25.42	-27.42	-26.40	-28.66	-29.10	
Average	-26.81 ± 1.46	-24.90 ± 0.98	-26.08 ± 1.28	-27.36 ± 0.60	-27.12 ± 0.84	-27.99 ± 0.88	-28.22 ± 0.82	

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Cocoa (*Theobroma cacao*) (Gattward et al., 2012) also represents the C_3 type plant range. However, the result is still tentative, thus, require further investigation using advance technique such as compound specific isotope analysis (CSIA).

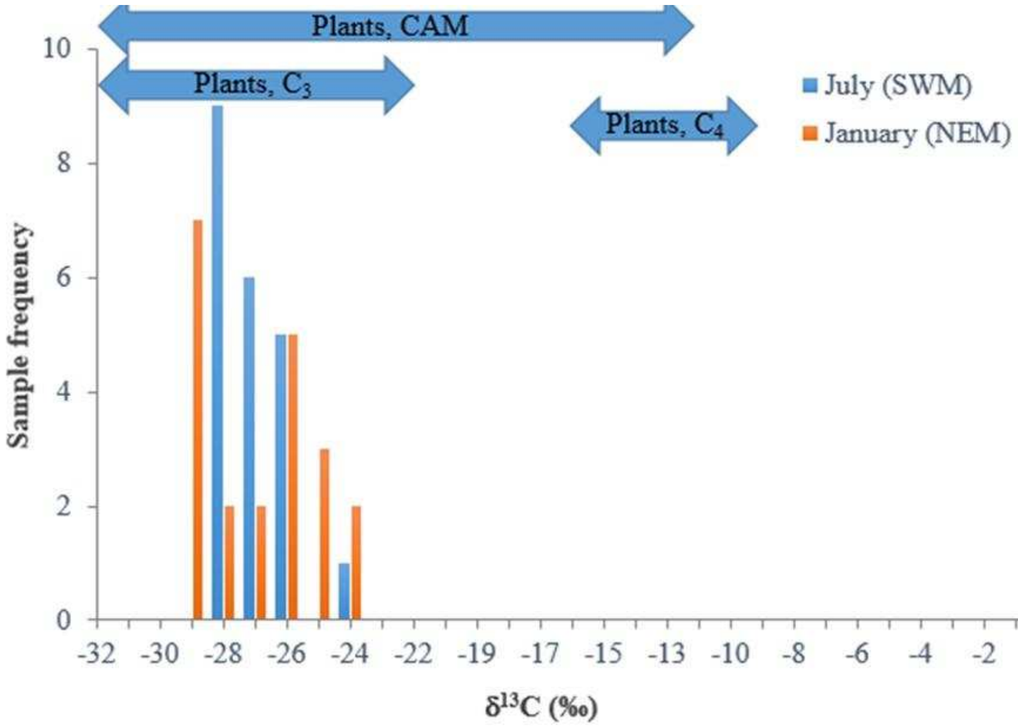

Figure 3. *Bar frequency plot δ^{13}C [‰] sediments in two different seasons*

The range of δ^{13}C could reflect sources of sedimentation, where most of the terrestrial plant that are present along the studied locations, are mainly from C_3 type plant. Theoritically, variations of C_3 values were due to kinetic isotope fractionation process, which includes microbial respiration of organic carbon, assimilation process of residual materials (Thornton and McManus, 1994; Teranes and Bernasconi, 2005) and changes in global carbon cycle (Oehlert and Swart, 2014), thus deposited in the water bodies.

The δ^{15}N sediment values at different study areas were presented in *Figure 4*, with information on organic content resulted from decomposition process. In general, the average value for δ^{15}N in July (SWM) and January (NEM) were +0.65 ± 2.44‰ and +3.35 ± 1.02‰ (*Table 2*) respectively. Both results of the two different seasons showed that δ^{15}N were ranged from -3.98‰ to +5.25‰ (*Table 2*), which demonstrated the dynamic of N cycle.

The average values of δ^{15}N for Brok, Betis, Nenggiri are 1.48 ± 2.48‰, 2.26 ± 1.82‰ and 0.70 ± 2.74‰ (*Table 2*), respectively, enriched in δ^{15}N. The upstream of Betis River is flowing from Lojing and merges with Brok River at the confluence of Nenggiri River. Richness of δ^{15}N indicates more nitrogen source mainly from anthropogenic activities (Dolenec et al., 2006; Brahney et al., 2014). The average values of δ^{15}N for Galas River, Pergau River and Lebir River are 1.51 ± 2.63‰, 2.50 ± 0.90‰ and 3.45 ± 2.63‰ (*Table 3*) respectively. Matured oil palm and rubber plantations are the major land use activities around the catchment areas.

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***Table 2.** Isotopic composition of stable isotope $\delta^{15}N$ in sediment*

Stable Isotope $\delta^{15}N$ [‰]								
	Rivers							Average season
Season	Broke	Betis	Nenggiri	Galas	Pergau	Lebir	Kelantan	
Jul (SEM)	1.41 -2.73 0.09	1.82 5.25 -0.29	-1.27 -3.98 1.92	1.25 -2.94 0.37	3.71 1.90 1.19	-1.45 2.33 4.82	-1.98 0.67 1.62	+0.65 ± 2.44
Jan (NEM)	3.31 2.96 3.82	2.42 2.82 1.54	2.89 2.47 2.19	3.47 4.48 2.42	3.21 2.62 2.40	4.87 5.03 5.14	4.17 4.31 3.81	+3.35 ± 1.02
Average	1.48 ± 2.48	2.26 ± 1.82	0.70 ± 2.74	1.51 ± 2.63	2.50 ± 0.90	3.45 ± 2.63	2.10 ± 2.49	

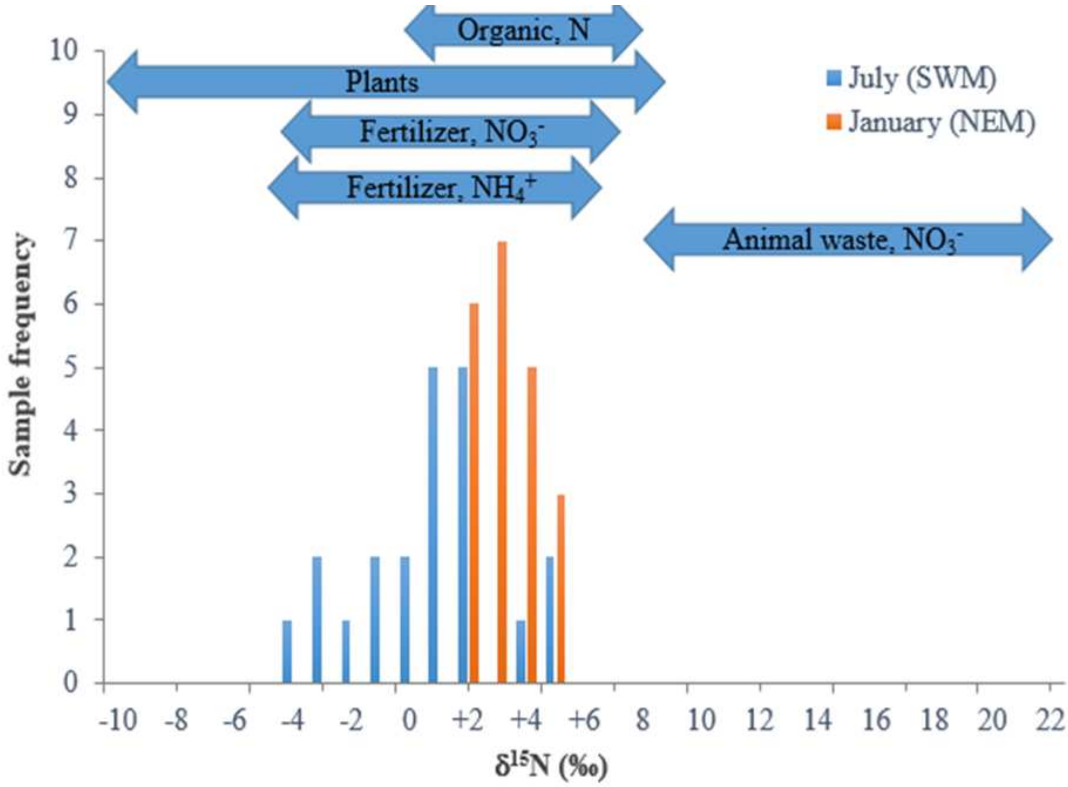

***Figure 4.** Bar frequency $\delta^{15}N$ [‰] sediments at two different seasons, Southwest Monsoon (July) and Northeast Monsoon (January)*

Kelantan river, which is situated at the downstream area, have average values of $\delta^{15}N$ 2.10 ± 2.49‰ (*Table 2*). The villages around the catchment may affect the nitrogen concentration in the sediments due to all the wash out from upstream river. Kelantan river exposed by many land use activities such as mining activity and development around the river area for new residential and towns, thus, posing significant impact on river sedimentation.

Distinct of $\delta^{15}N$ values are due to kinetic isotopic reaction and together with unidirectional reaction in hydrosphere cycle perform by bacteria activities (Heaton, 1986). The overlapping of $\delta^{15}N$ values reflect complex fractionation caused by multiple

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processes (mineralization, nitrification, plant uptake and denitrification) in nitrogen cycle (Lajtha and Schlesinger, 1986; Ryabenko, 2013).

Fertilizer appears as one of the main "fingerprints" in sediment samples. Both nitrate (NO_3^-) and ammonia (NH_4^+) are common results from industrial fixation of atmospheric nitrogen via measureable process of isotopic fractionation, whereby δ^{15}N depletes (Gunter, 1986). Meanwhile, organic nitrogen in soil undergo mineralization process that causes it to fractionate. It involves steps that can fractionate it and change δ^{15}N values in favorable condition with the aid of bacteria activities (Heaton, 1986).

The isotopic fingerprints of δ^{15}N (*Figure 4*) in sediments (+0 to +9) represent the Soil Organic Matter (SOM), suggesting significant erosion (due to land clearing) during the NEM (An et al., 2008; Chakravarty et al., 2012; Ickowitz et al., 2015).

C:N ratios in sediments

C:N ratio in sediments was analyzed to complement the terrestrial and organic matter sources characterized by δ^{13}C and δ^{13}N (Finlay and Kendall, 2007; Sanderman et al., 2015). *Figure 5* shows a biplot δ^{13}C and C:N ratio presenting various types of terrestrial and aquatic organic matter overlapping from sediment collections in Kelantan. There is absence of C_3 type plant from terrestrial source, which constitutes a ratio of more than 15. However, although the "fingerprint" of terrestrial plants might be present, in our case, the allochthonous component was not clearly identified, perhaps, due to the catchment settings (climate, hydrology, etc) that may speed up the rate of decomposition process in water body (McGill and Cole, 1981). Besides, the C:N ratio too can be changed due to degradation of organic matter during sediment diagenesis (Gao et al., 2012).

In overall, about 95% of C:N ratio were determined <12 (*Figure 5*), indicating the source of sedimentation particularly from aquatic part which is autochthonous (Tue et al., 2011). Even though it is believed that high turbidity and sedimentation in Kelantan catchment causes absence of aquatic macrophytes and seagrass, microalgae were present as autochthonous input. Besides that, it is evident that there are terrestrial, allochthonous input from the river being transported, which is phytoplankton (Tue et al., 2011). The C:N ratios value tend to decrease over time as degradation process release carbon dioxide (CO_2) or methane (CH_4), ammonia and other microbially-associated nitrogen (Gao et al., 2012). Additionaly, low of C:N ratio is caused by abundance of ammonium ions is absorbed into clay minerals (Rumolo et al., 2011).

C:N ratio shows higher in Nenggiri and Kelantan river with a slightly change in δ^{13}C range suggest that post depostional decay in organic sedimentary, might be due to rapid sedimentation process (Sanderman et al., 2015). From this biplot, it shows that the source of sediments was from a mixture of C_3 from terrestrial part, phytoplankton and algae. The input from outside is seemed superimpose to the large river, where it does not give such a vast impact to the ecosystem.

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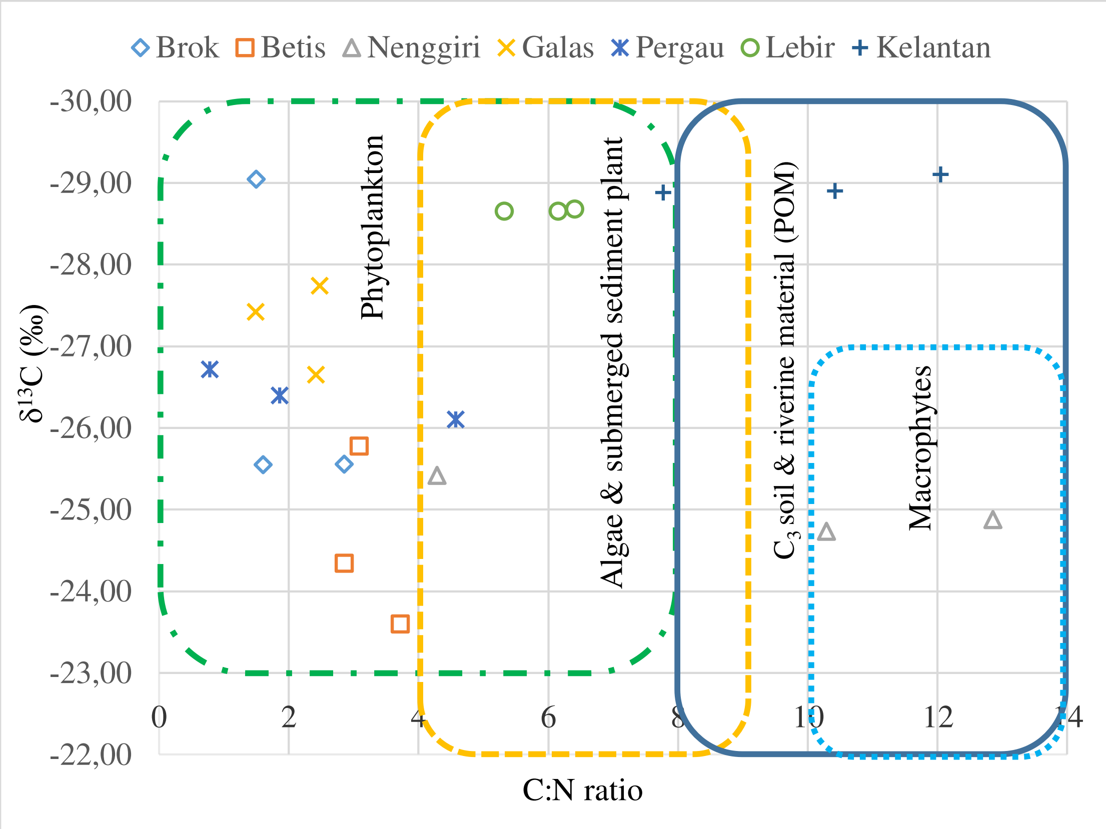

***Figure 5.** The $\delta^{13}C$ and C:N ratio of various types of terrestrial and aquatic organic matter overlap by the sediment range from Kelantan catchments*

Sediment accumulation in Kelantan river networks

Sedimentation in river is one of major flood factors in Kelantan's watershed. As mentioned in methodology, the sediment yield data were analyzed using multivariate principal component analysis (PCA) to determine major factors that are responsible for the sediment accumulation in water bodies. PCA is a technique to reduce large data into small variable number to summarize the data analysis (Pallant, 2001; Wuttichaikitcharoen and Babel, 2014).

***Table 3.** Proposed factor of sediment yields in Kelantan watershed*

Sediment yield	Kelantan catchment	F1	F2
	Component	All	Oct, Nov, Dec
	Eigenvalue	9.66	2.31
	Variability (%)	80.54	19.24
	Cumulative (%)	80.54	99.77
	Factor	Climate	Northeast Monsoon

PCA for sediment yields datasets (*Table 3*) shows that the main component for factor loading (*Table 4*) are characterized by two components with Eigenvalue > 1, which consist of all months throughout the year and component two only on October, November and December. This explains 99% (*Figure 6*) of cumulative variability respectively, reflecting seasonal factor whereby Northeast Monsoon in component

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factor two in agreement with Butt et al. (2011) and Hua (2014). The reason is because during this period, east coast states such as Kelantan receives extra rainfall, thus triggering erosion process, which results in sediments transport into the water.

Figure 6. *Scree plot Eigenvalue for sediment yield in Kelantan catchment*

Table 4. *Factor loadings of sediment yields over two principal component*

Months	F1	F
Jan	**0.96**	-0.27
Feb	**0.96**	-0.27
Mar	**0.96**	-0.26
Apr	**0.96**	-0.27
May	**0.97**	-0.25
Jun	**0.96**	-0.26
Jul	**0.97**	-0.25
Aug	**0.99**	-0.13
Sep	**0.89**	0.46
Oct	**0.67**	**0.74**
Nov	**0.72**	**0.70**
Dec	**0.66**	**0.75**

Moreover, sediments yield show significant results in Nenggiri Rivers, suggesting rampant land use activities which degrades the soil along the river banks. Logging and deforestation activities at upstream of Gua Musang is one of the evidences showing unsustainable land use activities that destroys the ecosystem services (Adnan and Atkinson, 2011). Anthropogenic activities such as deforestation and agriculture are the main factors of erosion process that contributes to sedimentation. Sand mining is also identified as one of the reasons that intensifies sedimentation in Kelantan river (Syahreza, 2012).

Water quality data (Principal Component Analysis, PCA)

Sediment transport have had tremendous impact on water quality of Kelantan river. A water quality set of data provided by Department of Environment (DOE) since 2004 to 2014 was analyzed by using PCA to determine major factors that responsible for the

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degradation of river networks in Kelantan catchment (Berok, Betis, Galas, Kelantan, Lebir, Nenggiri, Pergau).

PCA for water quality parameters datasets (*Table 5*) shows the main factor that plays a crucial role in determining the quality data of Kelantan catchment, which are characterized by two factor components with Eigenvalue > 1. According to Chatfield and Collin (1980) assumption, stated that components with eigenvalue less than 1 should be eliminated. This explains 51% (*Figure 7*) of total variance, due to anthropogenic factor, contributed by urbanization along the river (Ishak, 2014) with superimpose of natural factor.

Principal component analysis results show factor loadings in *Table 6*. Based on these parameters loading, the variables are grouped accordingly with their factors group. Factor loading with value more or equal than 0.60 were bold in the table below. Factor loading 1 consists of three parameters, which are Chemical oxygen demand (COD), Biochemical oxygen demand (BOD) and Suspended solid (SS). While Factor loading 2 are dissolved oxygen (DO) and pH unit followed by Factor loading 3 Ammoniacal nitrogen (NH3-N), Factor loading 4 dissolved oxygen (DO) and Factor loading 5 Suspended solid (SS).

Results showed that chemical oxygen demand (COD) is the most significant parameter in determining water quality of Kelantan river, which reflects the parameters to measure oxygen required to oxidize chemical substance through chemical process (Talib and Amat, 2012). According to Northeast Georgia Regional Development Center, (2001) COD values always have high value compared to BOD. This is because COD measurement only requires a few hours while BOD measurement can lead up to five days. Both COD and BOD are correlated process to each other as oxidation process that occurs during organic matter break down to a more stable form (Talib and Amat, 2012).

The second factor that contributes to COD value is phosphate concentration, as they are directly proportional to each other (Talib and Amat, 2012). The COD value will be high as phosphate concentration increases. The main source of phosphorus is mainly from agriculture fertilizer, manure industrial effluent and sewage. The major factor that causes high concentration of phosphorus in the river is soil erosion especially during flooding event (USGS, 2016).

Furthermore, as BOD is correlated with COD, it suggests that the other factor that influence BOD value is organic waste and detritus from terrestrial part, agriculture and also urban runoff, (Northeast Georgia Regional Development Center, 2001). These are also the same contributing factors of COD. Both COD and BOD play a major role in the deterioration of Kelantan's water quality.

Table 5. *Proposed factor of water parameter in Kelantan catchment*

Water quality	Kelantan catchment	F1	F2
	Component	COD, BOD, SS	pH, DO
	Eigenvalue	1.90	1.17
	Variability (%)	31.68	19.48
	Cumulative (%)	31.68	51.17
	Factor	Anthropogenic	Anthropogenic

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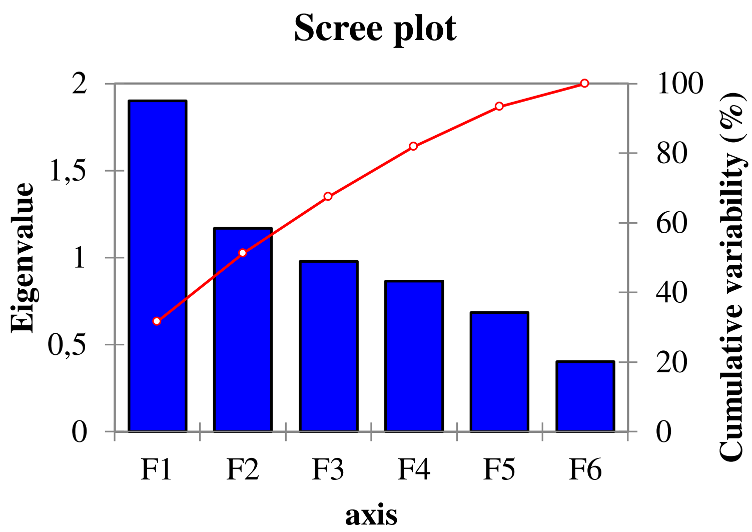

Figure 7. *Scree plot eigenvalue for water quality parameter*

Table 6. *Factor loading of river parameters over two principal component*

Parameter	F1	F2
DO mg/l	-0.32	-0.61
BOD mg/l	**0.74**	-0.39
COD mg/l	**0.84**	0.09
SS mg/l	**0.70**	0.07
pH Unit	-0.11	0.76
NH3-NL mg/l	0.20	0.24

Besides COD and BOD, suspended solids (SS) too, pronounces significantly, in agreement with stable isotopes results. This factor supports the sediment yield data, which was discussed earlier in the erosion mechanism. The other two parameters, which are dissolved oxygen and ammonical nitrogen are not the factors in determining the water quality of Kelantan river. Theoritically, DO depends on the water temperature (temperature depend on COD and BOD), sediment quantity in water flow and aeration. (Northeast Georgia Regional Development Center, 2001)

Conclusions

Stable isotope of $\delta^{13}C$ suggested that C_3 type plant are the dominant plants in Kelantan river with an autochthonous system as depicted in C:N ratio. However, it was evidenced through isotopic fingerprint of $\delta^{15}N$ and multivariate analyses of Water Quality and Sediment Yield datasets, anthropogenic factors have had significant impact on water quality and sedimentation of Kelantan river. Essentially, this research will help stakeholders to develop better strategies in restoring ecosystem services of Kelantan watershed. Balanced ecosystem, therefore, plays significant role in servicing the humanity, makes the ecosystem more resilience to natural disasters.

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ANOPLOPHORA CHINENSIS (FORSTER, 1771) (COLEOPTERA: CERAMBYCIDAE) REPORTED AT NEW LOCATION IN TURKEY

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Abstract. Invasive alien insect species have fast growth and reproduction, high spreading ability, tolerance tovery different enviromental conditions and ability to feed with many plant species. The increase in importing of the plants in the recent years has been causing the prensence of these species in Turkey. One of the these species, *Anoplophora chinensis* (Forster, 1771) (Coleoptera: Cerambycidae) citrus longhorned beetle was recorded for the first time in Marmara region, Istanbul, Turkey by Hizal et al (2015). This study is a second report for the presence of releated pest in Turkey. The new location (Bartin), in the western Black Sea region, shows that the insect is expanding its distribution area in Turkey.

Keywords: *Anoplophora chinensis, citrus longhorned beetle, Cerambycidae, new location, Turkey*

Introduction

The genus *Anoplophora* (Coleoptera: Cerambycidae) consists of 36 species of longhorned beetles indigenous to the temperate and tropical regions of Asia (Lingafelter and Hoebeke, 2002). Among these species, the citrus longhorned beetle, *Anoplophora chinensis* is a poliphagous woodboring beetle native to Eastern Asia with a host range quite wide causes damage on more than 100 species of trees and shrubs (Peverieri et. al., 2012; EPPO, 2013a). The beetle gets its name from the damage caused to citrus groves in its native China. Unlike many cerambycids that primarily attack dead trees, this beetle attacks apparently healthy trees and sever tissues that carry nutrients, water, and subsequently kill the host tree (Chambers, 2002; Lance, 2002).

The citrus longhorned beetle has been introduced to Europe on several occasions. It was first discovered in Europe in 2000 at Parabiago, Italy and respectively it has been recorded in Netherlands and France (2003) and Switzerland (2006) according to FAO (2009). In Italy more than 18000 plants have been removed for the eradication program and the cost has amounted to about 12 million euros (Jucker and Lupi, 2011). Adults of *A. chinensis* were captured on 12 June 2014 in Sile region of Istanbul province and it was given as a new record to Turkish invasive alien insect species fauna by Hizal et al. (2015).

A. chinensis is a new threat on a wide range of broadleaved trees and shrubs in Turkey. Therefore its distribution areas and the behaviour of the pest in these areas should be investigated.

Materials and Methods

A total number of 28 adult samples were collected from a private nursery in Bartin region on ornamental plants consisting of *Acer palmatum purperea, A. negundo flamingo* and *A. platanoides* by a Japanese umbrella. Specimens were photographed with Samsung Pro-815 digital camera and they were examined under the Olympus

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SZX7 stereomicroscope. The identification process was carried out using specific literature (Lingafelter and Hoebeke, 2002; Gyeltshen and Hodges, 2005; EPPO, 2013a). The samples are stored in the collection of Bartin University, Faculty of Forestry, Department of Forest Entomology and Protection.

Results and Discussion

Experimental location: Turkey, Bartin, 41˚ 38' 17" N- 32˚ 19' 48" E, 13m, 15.VI. 2014 1♂, 17.VI. 2014, 5 ♂, 10 ♀, 19.VI. 2014, 3 ♂, 9♀

Morphology

The beetle is large, stout, and approximately 21 to 37 mm long with shiny black elytra marked with 10 to 20 white round spots (Lingafelter and Hoebeke, 2002). Generally males are smaller than females, and have their abdomen tip entirely covered by the elytra, in contrast to the partially exposed abdomen of females. Also, the male elytra are distally narrowed compared to the rounded female elytra. Another difference between males and females are antennal sizes. The male's antennae are approximately twice as long as its body compared to the female's antennae which are only slightly longer than the body. Each segment of the long, 11-segmented antennae is basally marked with white or light blue bands (Gyeltshen and Hodges, 2005). The anteriorly and posteriorly narrowed pronotum has a pair of stout spines extending from its sides and some with two blue and white tufts of hair on pronotum (EPPO, 2013a). The base of the elytra has numerous short tubercules/granulae, a morphological character that may help to differentiate *Anoplophora chinensis* from the, *A. glabripennis* (Gyeltshen and Hodges, 2005) (*Figure 1 A-D*).

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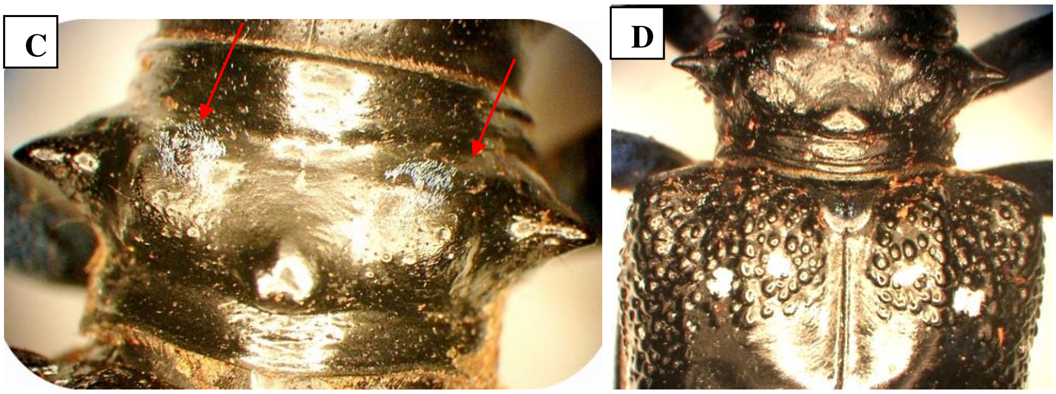

Figure 1. *A. Adult of Anoplophora chinensis B. Antenna with blue-white bands C. Pair of stout spines and two blue-white tufts of hair on pronotum D. Tubercules/granulae on the base of the elytra*

Distribution: The species has been reported China, Croatia, Germany, Guernsey, Hong Kong, Indonesia, Italy, Japan, Korea, Lithuania, Malaysia, Myanmar, Philippines, Switzerland, Taiwan and Vietnam (EPPO, 2014).

Host plants: *Acer* spp., *Aesculus hippocastanum*, *Alnus* spp., *Betula* spp., *Carpinus* spp., *Citrus* spp., *Cornus* spp., *Corylus* spp., *Cotoneaster* spp., *Crataegus* spp., *Fagus* spp., *Lagerstroemia* spp., *Malus* spp., *Platanus* spp., *Populus* spp., *Prunus laurocerasus, Pyrus* spp., *Rosa* spp., *Salix* spp., *Ulmus* spp., *Casuarina* spp., *Cryptomeria* spp., *Ficus* spp., *Hibiscus* spp., *Litchi* spp., *Mallotus* spp., *Melia* spp., *Morus* spp. and other woody plants (EPPO, 2013b).

In the study, adults of *A.chinensis* were detected in private nursery on maple trees (*Acer palmatum purperea*, *A. negundo flamingo* and *A. platanoides*) that imported from China. These beetles were recorded in the same time as it was recorded first time in Istanbul by Hizal et al. (2015). Due to its polyphagous character, host plant is considered under high risk of attack, especially *Acer* species. The pest is a serious problem for the nursery industry, in the production of ornamental trees; it is also a potentially pest of citrus orchards and of many other deciduous trees. Adults feed on the fresh bark of small twigs and branches, and sometimes on leaf petioles. The females also chew from the bark of the host tree to the cambial layer, forming 'egg scars'; then inserts her ovipositor and lays a single egg (Lingafelter and Hoebeke, 2002). Larvae feed and develop in the wood of the main roots and trunks, where they create tunnels. Exit holes have been found only at the base of trees (*Figure 2 A-D*). Heavy infestations can kill the host trees (Maspero et al., 2005).

This species, is placed at the list of 'Unknown presence and harmful organism for importing' as a part of Agricultural Quarantine Regulations Bylaw -1 An Obstacle Harmful Organism for Importing which is prepared in terms of the law no. 5996 Veterinary Services, Plant Health, Food and Feed promulgated on 13 June 2010 on Official Gazette. In addition, as regard to European Union *Anoplophora chinensis* commission decision of 1 March 2012 and law no. 2012/138/EC, specifically, by law is prepared and on 17 June 2014 law no. 29033 promulgated on Official Gazette and entered in force (Anonymous, 2014).

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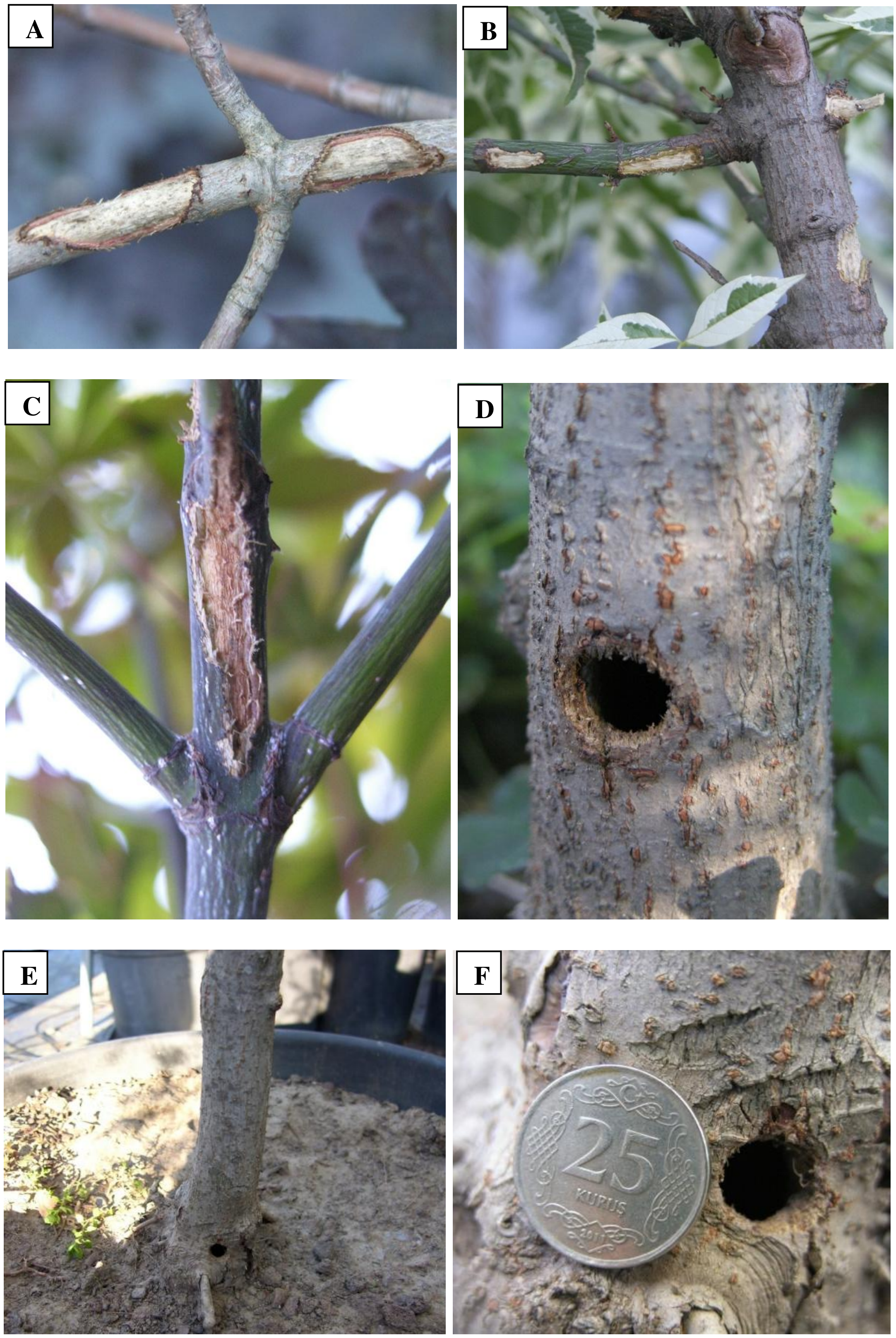

Figure. 2 *A-C. Feeding damage of adults D-F. Exit holes*

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Most of the known host plants of the citrus longhorned beetle exist also in Turkey. With respect to this, discovering of the releated invasive pest and exploring its new distribution areas in Turkey has a very important value for management of Turkish forests. Ayberk et al. (2014) has reported another invasive longhorned beetle, the Asian longhorned beetle, for the first time in Turkey. The authors has reported the adults of *Anoplophora glabripennis* were collected from Istanbul province (Zeytinburnu) damaging on *Acer negundo* trees in July, 2014. For this purpose, as the first step should be to determine real status of invasive *Anoplophora* species in Turkey. And then to eliminate these extremely dangerous quarantina pests is to remove infested trees and destroy them by chipping or burning immediately. To stop the spread of the citrus and Asian longhorned beetles, it should be to follow the quarantine procedures strictly in infested areas.

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A REVIEW OF COMMUNITY-BASED NATURAL RESOURCE MANAGEMENT

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Abstract. This paper reviews initiatives of Community-Based Natural Resource Management (CBNRM) from 21 case studies in different countries. The study uses Ostrom's design principles and eight objective measures. These are empowerment, public participation, equity, conflict resolution mechanisms, similarities between misuse and establishment of rules for indigenous environments, effective monitoring, collective choice arrangements and sanctioning activities for the use of common resources to evaluate and compare the case studies to examine the cases. Based on the analyses, successes and failures of CBNRM were determined. Successes were evident in the management of water resources in Honduras, India, Kenya, Nepal and St Lucia. CBNRM was also successful in Fiji, in joint forest management in India and other instances such as in Alaska, China, Cambodia, Namibia, Malaysia, Mexico, Washington, and to a small extent in Papua New Guinea. However, CBNRM initiatives failed in managing wildlife conservation in Nepal and Kenya, and in Tanzania wildlife management, Uganda and Zimbabwe. CBNRM failure was attributed to the uneven distribution of the benefits of natural resources, lack of empowerment, low community participation, failure to resolve conflicts, among many factors. In this study, the recommendations is made that similar research should be conducted with a larger sample size and employ other techniques such as Principal Component Analysis for examining the characteristics that achieve effective and sustainable CBNRM initiatives.
Keywords: *community, participation, biodiversity, water management, sustainability, international comparison*

Introduction

The worldwide failure of centralised approaches to managing natural resources led to the search for a viable and sustainable alternative approach by conservationists to achieve sustainable management (Nabane and Matzke, 1997). The approach whereby local communities are given ownership rights to manage natural resources became common in the 1960s when it was named community-based natural resource management (CBNRM). It is also called community-based conservation. According to Songorwa (1999), CBNRM aimed to create conditions under which most members of the community stood to benefit from the sustainable utilisation and management of wildlife resources. This would occur through a bottom-up participatory approach (Songorwa, 1999) based on a number of principles. These principles, which include meeting the basic needs of local people, putting resources under local control rather than the control of the state government, obtaining equal delivery and apportionment of socio-economic benefits and resources, and commitment involving members of the community and the local institutions in managing and conserving natural resources. This would be achieved regardless of their gender and would encompass the defence

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and legitimisation of local resources and property rights. There would also be a willingness to embrace traditional values and ecological knowledge in the management of present resources and a need to associate and resolve the objectives of social and economic growth for the protection and conservation of natural resources in the environment (Songorwa, 1999; Kellert et al., 2000). Also, CBNRM attempts to integrate the goals of conservation, sustainable development and community participation (UNDP, 2012c). This is performed by applying Ostrom's 1990 design principles (*Table 1*) on governing the common elements concerning empowerment, public participation, equity, conflict resolution mechanisms, congruence between appropriation and provision of rules for local conditions, effective monitoring, collective choice arrangements and sanctioning activities for the use of common resources. Ostrom design principles were based on extensive studies of long-enduring governing institutions of common-pool resource management across different systems such as wildlife, fisheries, and forests (Ostrom 1990, 2009; Dell'Angelo et al., 2016). The eight design principles that were identified by Ostrom (1990) include opportunities for collective choice and local self-determination, approaches to monitoring, congruence with local conditions, sanctions, and conflict resolution. It included the incorporation of multiple, nested layers of organisation, shows the best practices, and describes the rules and structures of robust institutions associated with the sustainable governance of common pool resources. This study employed Ostrom's (1990) design principles for natural resource management as a diagnostic framework to analyse the CBNRM initiatives in the 21 case studies.

Table 1. *Ostrom's 8 design principles for natural resource management of common-pool [Adapted from Ostrom (1990)]*

Ostrom's design principles	Operationalisation
1. Clear boundaries	Individuals or households who have the right to use the common-pool resource are clearly defined
2. Congruence with local conditions	Rules restricting time, place, technology, and quantity of resource use are well adapted to local conditions
3. Collective-choice arrangements	Most individuals affected by the rules can participate in modifying them
4. Monitoring	Common-pool resource conditions and use are monitored by the users themselves or by people accountable to the users
5. Graduated sanctions	Users who violate resource-related rules are likely to be assessed penalties that correspond to the seriousness and context of the offense

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6. Conflict-resolution mechanisms	Users and officials have rapid access to low-cost local arenas for resolving conflicts among users and conflicts between users and officials
7. Recognition of the right to organization	The rights of users to devise their own organisations are not challenged by external government authorities
8. Nested governance	Appropriation, provision, monitoring, enforcement, conflict resolution, and governance are organized in multiple, nested layers

CBNRM as a concept emerged and gained popularity in the early 1980s as an alternative to resource regimes that were perceived to be failing (Gibson and Mark, 1995; Matzke and Nabane, 1996). CBNRM has been extensively promoted in recent years as a method for investigating natural conservation and socio-economic goals. Examples such as forests, wetlands and grasslands are among several projects that involve local communities in managing natural resources in protected areas. Despite variation in forms of CBNRM such as the management by communities of wildlife, grassland, forests, water resources and many others, they share common features, including the involvement of local people in the management of their resources. This implies a willingness to devolve power and authority from central government to local institutions and people. This happens because of their belief and desire to integrate traditional ecological knowledge in balancing socio-economic and environmental goals in the conservation and protection of natural resources (Kellert et al., 2000).

CBNRM syntheses that has been conducted (Agrawal and Gibson, 1999; Allan and Curtis, 2005; Barkes, 2004; Campbell and Vainio-Mattila, 2003; Child, 2007; Deker et al., 2002; Gruber, 2010; Kellert et al., 2000; St. Jacques, 2009; Measham and Lumbasi, 2013) has been consistent with Ostrom's 1990 design principles for governing the common elements. Agrawal and Gibson (1999) proposed focusing on institutions rather than the community if CBNRM programmes are to be successful. Allan and Curtis (2005), in their study on adaptive management, found that using both passive and active adaptive management may be inhibited by deeply rooted social norms and institutional structures. Barkes (2004) proposes the need for greater consideration of the nature of people, communities and institutions, and how they interrelate at several levels in CBNRM programmes. In their synthesis of CBNRM, Kellert et al. (2000) note that the success of a CBNRM programme may depend on the structure of institutions, socio-economic development and scientific considerations. They also observed that the main and consistent obstacle was a failure to control and monitor the behaviour of complex organisations, mostly bureaucratic and local institutions. Measham and Lumbasi (2013) further observed that communities with high level of ownership tend to have effective CBNRM programmes.

Assistance in the analysis of the current state of CBNRM initiatives was provided by Gruber (2010) who tried to develop wide organisational principles and main characteristics of effective and sustainable CBNRM. However, he could not recognise the key characteristics that were most critical in attaining long-term effective and

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sustainable CBNRM. Further, in his CBNRM synthesis, St. Jacques (2009) observes that participation needs to be flexible, not only to meet project phase objectives but also to allow for content-specific needs. He also observes that the most democratic form of participation is one that facilitates social learning and maximises the opportunity for information flows between stakeholders. Campbell and Vainio-Matilla (2003) also note in their study that lessons learned in participatory development have not been used in community-based conservation. This was partly because of the different emphasis on means versus ends in participatory development and community-based conservation. Furthermore, scholars such as Brooks et al. (2013) observed that Community-based conservation promoted the idea that long-term conservation success required engaging with and providing benefits for local communities. This was in line with Gurneya et al. (2016) who found that individuals' level of participation in marine protected areas (MPA) management was related to socioeconomic factors. In short, success in conservation is often predicated by local support for conservation which is influenced by perceptions of the impacts that are experienced by local communities and opinions of management and governance (Bannett and Dearden, 2014).

This review paper examined critically the success and failure of CBNRM that occurred in various case studies from 21 different countries based on the eight design principles developed by Ostrom (1990) that are displayed in *Table 1* above.

Methods

To determine the relative success of CBNRM of the 21 case studies from the different countries, each variable was first given a score that was entered into a spread sheet. The scores were either 0 for no evidence or evidence not deemed useful or 1 for the existence of evidence provided in the case study. Second, the scores were then added to give a total value out of 8. The higher the number, the greater was the relative success of the CBNRM initiative in each of the resources being managed in the 21 case studies. Third, the cross-tabulation analysis was performed using the variables scored in the spread sheet. Fisher's Exact Test in the cross-tabulation was used to analyse the relationship between each variable and the success of CBNRM. Fisher's Exact Test was appropriate for this study because of the small sample size used (21 cases). Data reduction techniques such as principal component analysis (PCA) could therefore not be used as it is recommended for a sample size of at least 50 and above (Hair et al., 2010).

Results

In *Table 2*, the countries where the case studies were done, as well as the name of the case study are shown. An indication is also given of the resource being managed and the source of data where the case study was reported. The resources managed in the case studies analysed included water, forest, wildlife, fish and wetlands. The case studies examined were drawn from different countries. *Table 3* indicates the CBNRM performance criteria and the totals for the various case studies analysed. The CBNRM performance criteria analysed in the study include equity, empowerment, community participation, monitoring of biological diversity, conflict resolution, collective choice, and local condition rules. *Table 3* indicates the total score for CBNRM performance criteria in each country, too.

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Spread sheet scores for cases studies examined are shown in *Table 3*. The table shows that for cases from Honduras up to Namibia, the total score for all the variables was 8, while Alaska, Washington, China and Papua New Guinea scored 7, but Botswana scored 2, with Nepal, Kenya, Zimbabwe and Tanzania scoring 0 for all the eight variables examined.

Table 2. *Country of case study, type of case study type of resource managed and source of data*

Country	Name of case study	Resource managed	Data source
Honduras	Community-based water supply Pasos 111	Water	St Jacques 2009
India	Holistic watershed management in Sukhomojri	water	Islam and Jain 2011
Kenya	Mara river water association	Water	UNDP 2012a
Nepal	Irrigation agriculture sector project	Water	Islam and Jain 2011
St Lucia	Water catchment project	Water	St Jacques 2009
Fiji	Fiji Locally managed marine area network	Water	UNDP 2012d
India	Joint forest management	Forest	D’silva and Nagnath 2002
Mexico	Conservation forest management		Brey et al., 2003
Malaysia	Regional awareness of Cameroon Island	Water	St Jacques 2009
Cambodia	Monk community forest	Forest	UNDP 2012 b
Namibia	Conservancy and wildlife management	Wildlife	Jones 1999
Alaska	Cooperative management of North American Pacific salmon	Fish	Kellert et al., 2000
Washington, DC.	Cooperative management of North American Pacific salmon	Fish	Kellert et al., 2000
China	Kanghua community development centre	Forest	UNDP 2013
Papua New Guinea	Sepik wetlands management initiative	Wetlands	UNDP 2012e

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Botswana	CBNRM in Okavango Delta	Wildlife	Mbaiwa 2012
Nepal	CBNRM in Annapuma and Makalu – Barun	Wildlife	Kellert et al., 2000
Kenya	Kimana community wildlife sanctuary	Forest	Kellert et al., 2000
Zimbabwe	Impact of CAMPFIRE on local community	Wildlife	Mutandwa and Gadzirayi 2007
Uganda	Wildlife conservation around Mburo national park	Wildlife	Emerton 1999
Tanzania	Wildlife management in **Serengeti**	Wildlife	Emerton and Mfunda 1999

Source: Compiled from literature, 2014

Table 3. *Performance of CBNRM criteria and totals for the various case studies*

Case study	**CBNRM performance criterion**								**Total score**
	Equity	**Empowerment**	**Participation**	**Monitoring/biological diversity**	**Conflict resolution**	**Collective choice**	**Local condition rules**	**Sanction activities**	**Max score = 8**
Honduras	1	1	1	1	1	1	1	1	8
India water case	1	1	1	1	1	1	1	1	8
Kenya water case	1	1	1	1	1	1	1	1	8
Nepal water case	1	1	1	1	1	1	1	1	8
St Lucia	1	1	1	1	1	1	1	1	8
Fiji	1	1	1	1	1	1	1	1	8
India joint forest case	1	1	1	1	1	1	1	1	8
Mexico forest case	1	1	1	1	1	1	1	1	8
Malaysia water case	1	1	1	1	1	1	1	1	8

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Cambodia	1	1	1	1	1	1	1	1	8
Namibia wildlife	1	1	1	1	1	1	1	1	8
Alaska goose	1	0	1	1	1	1	1	1	7
Washington fisheries	1	0	1	1	1	1	1	1	7
China wildlife	1	0	1	1	1	1	1	1	7
Papua New Guinea	1	0	1	1	1	1	1	1	7
Botswana wildlife	1	0	1	0	0	0	0	0	2
Nepal wildlife	0	0	0	0	0	0	0	0	0
Kenya wildlife	0	0	0	0	0	0	0	0	0
Zimbabwe wildlife	0	0	0	0	0	0	0	0	0
Nepal wildlife	0	0	0	0	0	0	0	0	0
Tanzania wildlife	0	0	0	0	0	0	0	0	0

Cross-tabulation of the variables and CBNRM are shown in *Appendix 1*. The cross-tabulation analysis was performed using scores from the spread sheet. The Fisher's Exact Test in the cross-tabulation reviews the relationship between each variable and the success of CBNRM. The Fisher exact test value = 17.008 is significant at 5% level ($p < 0.05$). We therefore reject the null hypothesis that there is no association between CBNRM and equity. This shows that for CBNRM initiative programme was successful, and equity needs met. From the cross tabulation *Table 3* of CBNRM versus equity, it can be seen that if equity is absent, then CBNRM is not achieved. However, the table further shows that CBNRM can only be successful if equity is partially or fully achieved. The Fisher exact test is significant for the subsequent test of association between CBNRM and other variables examined. All the variables are important if CBNRM initiate is to be achieved (*Table 3*). For the entire cross tabulations for the variables assessed, Fisher exact test value is 0.00 indicating that all the variables need to be achieved to have a successful CBNRM initiative.

Discussion

We analysed CBNRM initiatives in 21 case studies from different countries using Ostrom's (1990) eight design principles of natural-resource management and eight

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objective measures. We found that some CBNRM initiatives show that their institutional structures reflect the eight design principles while others are not as discussed below. Those that were aligned with the design principles were successful while those that were not were unsuccessful. This is discussed more as follows.

Gender and equity

CBNRM advocates equal opportunities for men and women in natural resource management. Involving women in natural resource management programmes leads to positive outcomes, as women have a key influence on the environment (Songorwa, 1999). Gender balance has resulted in the success of most CBNRM initiatives where this was maintained. For example, the involvement of women in the management of forests in India led to success in that the first president of Vana Samarakshana Samith (VSS) is a woman and half of the members of the managing committee of VSS are women, too. The high involvement of women in India's forest management is attributed to the local demography of the area, which favours women (D'silva and Nagnath, 2002; Pathak and Gour-Broome, 1999). In Cambodia, a case study in the Monk Community Forest shows that women are actively encouraged to participate in CBNRM programmes; they are encouraged to take part in increasing awareness about activities in villages and to participate in the seven village sub-committees (UNDP, 2012a). In Mexico, there is strong participation of women in the management of activities in forests, such as bottling of water and tapping of resin (Bray, 2003). Gender balance is also a priority in the Fiji case study about a locally managed marine area network. There is a gender programme in which meetings are held with local women's groups that are encouraged to discuss the progress of the CBNRM initiative. This empowers women to make decisions on the management of their natural resources.

In the Honduras case study, dealing with the community-based supply of water and sanitation, sustainability is demonstrated through capacity building of the local community in water management, as water committees are established in the area. It is important to note that representation by women involved in decision-making did increase to 30% of the total number of individuals participating in the process of management (St. Jaques, 2009). This has led to an improvement in access to potable water and sanitation services in the area.

Some CBNRM initiatives, however, pays less attention to gender balance in their management of natural resources. This is the case in Zimbabwe's CAMPFIRE programme, where the participation of females is low (Mutandwa and Gadzirayi, 2007). Furthermore, in Nepal and Kenya women are marginalised (Kellert et al., 2000). In Botswana, Uganda and Tanzania the case studies did not provide information on how gender was addressed in their CBNRM initiatives.

Equal distribution of natural resource benefits to resource users motivates them to manage their resources sustainably. In Cambodia motivation to protect the forest emanates from the material benefits the forest offered to the local people, which are distributed equally to all the resource users (UNDP, 2012b). In Washington, the CBNRM initiative is a success because of the benefits from the natural resources that are distributed equally, which is evident from the lower number of conflicts that are registered in the case study (Kellert et al., 2000). When natural resource benefits are not distributed equally to all resource users, it tends to lead to conflicts. In Papua New Guinea, the CBNRM initiative provides benefits to the community in the form of economic benefits from the wetland resources hence it is a success (UNDP, 2012d). In

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India, local communities have equal access to forests and have employment opportunities in the forest work, therefore, they are motivated to protect the forest (Pathak and Gour-Broome, 1999). In their study on the effect of equity in benefit sharing in Nepal community forest programme, Luintel et al. (2017) noted that equity has been crucial in motivating forest managing communities.

In some case studies, however, equal distribution of natural resource benefits to all beneficiaries is a failure, and this leads to deficiencies in managing the natural resources effectively and in a sustainable manner. For example, case studies from Kenya, Nepal, Tanzania, Uganda and Zimbabwe reflect the unequal distribution of natural resource benefits. In Kenya, only a small minority received monetary benefits, while in Nepal there is uneven allocation of natural resources. This is because local people living closer to CBNRM headquarters receive more development benefits than those who live further from the headquarters in both Annapurna conservation areas (ACA) and Makulu barun conservation area (MBCA) (Kellert et al., 2000). In Zimbabwe's CAMPFIRE programme, the local community receives no economic benefits from their natural resources (Mutandwa and Gadzirayi, 2007). In Botswana unequal distribution of benefits occurs, as only 40% of the locals benefit from the wildlife resources (Boggs, 2000). Failure to distribute resource benefits equally to all beneficiaries leads to conflicts in the community and also reduces interest in managing natural resources effectively.

Monitoring

Ostrom's 1990 design principles advocate effective monitoring procedures in the management of common pool regimes. In the Mexico case study, effective monitoring is demonstrated by the expansion of canopy from 1982 to 1993, where the communities place four hectares under strict protection for the conservation of the endangered species Hickel's fir (*Adies hickelii).* The certification of 502,656 ha of forest in 2002 in 25 communities under the criteria of the forest stewardship council and the declaration of 500,000 ha in 1980 by the state of Quintana Roo as permanent forest areas demonstrates an increase in re-afforestation by the community, with limits being placed on agricultural activities in the forest area. Other monitoring indicators in Mexico include prohibiting all hunting activities in forest areas and willingness to reduce the logging volume in the Quintana Roo community and Laguna Kana' from 29% and 37% respectively (Bray, 2003). Effective monitoring procedures are also observed in Puget Sound in the State of Washington where ecological information on various salmon stocks has improved, and coordination of conservation efforts is enhanced among stakeholders (Kellert et al., 2000). The same happened in India, where a reduction in the demand for firewood is noted (D'silva and Nagnath, 2002). In the case of community water resources management in India, the programme has an ecological impact, namely an increase in the availability of water, as several rivers became perennial. There is also an increase in agricultural productivity where wheat yields are doubling (Islam and Jain, 2011).

However, in some case studies, there are no effective procedures to monitor natural resources. In Kenya for example, there is insufficient monitoring of observations of the wildlife resource because patrols by game scouts are highly sporadic. Data collected on the wildlife population and their habitats and on ecologically threatened and endangered species are insufficient. Moreover, little data collection on the dynamics of wildlife populations in the area and encroachment on the habitats of wildlife resources in parts

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of the Kimani community wildlife sanctuary (KCWS), take place (Kellert et al., 2000). In Nepal, a case study in the Annapuna and Makalu-Barun areas reveals that little time is being devoted to monitoring and protecting biological diversity, as most of the work is focused on building and local communities (Kellert et al., 2000). There is also insufficient monitoring of wildlife resources in Botswana and Zimbabwe, as no information is available to indicate the dynamics of resources in the study areas (Mbaiwa, 2012). The picture is similar in Tanzania and Uganda for their wildlife management case studies.

In summary, gender balance, equity and effective monitoring of natural resources are important for the success of a CBNRM initiative. This is because they all lead to the sustainability of natural resources.

Devolution and empowerment

Design principles developed by Ostrom in 1990 also advocate defined membership and rights in the management of common pool regimes. This is because devolution empowers resources (Armitage, 2005; Child, 2007; Grumbine, 1994). Measham and Lumbasi (2013) observe that communities with high level of ownership tend to have effective CBNRM programmes. Therefore, local communities will only be empowered if devolution is fully achieved. Empowering local communities builds commitment in the users of natural resources to manage such resources effectively (Bannett and Dearden (2014). Unfortunately, where local communities are not empowered to manage their natural resources, they usually remain unmotivated, and this has a negative effect on the well-being of the natural resources that are available. In all the case studies that are examined, attempts are made to devolve authority from the state to the local power, but the effectiveness of that devolution is variable. For example, in Kenya devolution of authority usually results in power being concentrated in certain groups or members, with others being excluded, and in Nepal people of a low caste and women are under-represented in conducting the management of natural resources (Kellert et al., 2000). Empowerment is also lacking in Zimbabwe's CAMPFIRE programme, where Mutandwa and Gadzirayi, 2007) observes partial devolution of natural resources and the exclusion of the local people in decision-making and management. In Botswana, too, despite job creation related to wildlife activities, no empowerment is seen among the local people (Mbaiwa, 2012), as the management of the resources is entirely in the hands of the government (Boggs, 2000).

In Fiji, the locally managed marine area within the network was established in 1997 in the Ucinivanua community, where the local community are empowered through the building of on-going capacity with the necessary knowledge to reverse the decline in their natural resources. Also, the emphasis is placed on the importance of collecting data as a tool for learning, alongside on-site training workshops and encouragement in the use of adaptive management as a key to achieving best practices. Moreover, a network has been developed that recognises the autonomy of the local communities' management of their marine resources, while providing support and guidance to help them achieve the best results and take responsibility for planning and facilitating the programme, while decision-making, implementation and evaluation are undertaken on ground level by the individual groups (Gruber, 2010).

The situation concerning empowerment is different in Alaska, Cambodia, China, Fiji, India, Japan, Malaysia, Mexico, Namibia, Papua New Guinea, St Lucia and Washington. In Alaska's Kuskokwim River watershed the local community is

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empowered to manage their salmon resource. In Cambodia, the programme for the management of natural resources has empowered the participants to have a voice in the management of the community forest through their representatives on the central management committee and the sub-committees (UNDP, 2012b). In China, the Kanghua community placed a strong emphasis on ownership of the natural resource by the local people, giving the community a strong sense of engagement and commitment that leads to the success of the initiative. The management programme for sika deer in Japan also empowers the local community, as they are involved in the decision-making process through the Nishiokoppe Wildlife Steering Committee (Decker et al., 2002). The same happens in Cambodia, where the participants have a voice in the management of the community forest through their representatives on the central management committee and the sub-committees. The importance of empowerment is further demonstrated by the Malaysian case where the World Wide Fund for Nature (WWF) withdrew its support in 2004. They also withdrew funding for the initiative of the Regional Environmental Awareness of Cameroon Highlands (REACH). Thereafter, funding was entirely depended on membership fees, donations and fundraising ventures (St Jacques, 2009). This confirms the empowerment of the local community to manage their resources by a non-governmental organisation because the initiative continues even after support is withdrawn.

In Mexico, devolution of responsibility for forest resources to the local communities is about 80% (Bray, 2003). The Mexican case also clearly shows that large numbers of communities are managing common property forests for commercial production of timber and finished timber products in some areas in Mexico, unlike in less developed countries where community forest management usually entails the management of non-timber forest products or wood for domestic use on government land (Bray, 2003). In St Lucia's water catchment project, community empowerment is demonstrated by the full participation of the local community and through capacity building in the various awareness programmes that are offered. Examples of this are technical training sessions and exchange programmes with similar community-based organisations, both in St Lucia and elsewhere (St Jacques, 2009). In Washington, the indigenous people are empowered through a partnership with the fisheries department, which gives them sufficient legal rights and authority to manage their local fisheries (Kellert et al., 2000).

Conflict resolution

Cheap or accessible conflict resolution mechanisms are one of the key principles designed by Ostrom (1990) in the management of common pool regimes. Conflicts in CBNRM programmes cannot be avoided, as management involves many stakeholders who have an interest in the natural resources. It is therefore important for CBNRM to factor in conflict resolution strategies at the start of the programmes (Ostrom, 1990). In the cases analysed, conflict resolution is addressed well in all of the water cases mentioned. In case studies of wildlife management in Kenya, Tanzania, Botswana, Zimbabwe China, and Nepal, however, conflicts are rarely resolved. For example, in all of these case studies, natural resource conflicts frequently occur, although the sources of conflict vary from place to place. In Kenya conflicts arise from wildlife depredation and disputes with neighbouring group ranches over boundary delineations and those related to institutional posts (Kellert et al., 2000. These cases are rarely resolved by management in the area, and little or no compensation is provided for damage caused by wildlife. In Nepal, the causes of conflicts are mostly related to power struggles among

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members and members of the institutions, especially the elite. Local conflicts in institutions also occur in the area as a result of overlapping jurisdictions and mandates (Heines and Mehta, 1999). In Tanzania, conflicts did arise from serious crop damage, usually caused by wildlife in the study area, but no compensation is paid for the damage that occurs (Emerton, 1999). The case is the same in Botswana and Zimbabwe, where community conflicts are on the increase but rarely resolved and no compensation for wildlife damage is paid (Mutandwa and Gadzirayi, 2007; Mbaiwa, 2012). Empowerment and conflict resolution lead to the sustainability of natural resources, and therefore CBNRM programmes could be successful.

Participation

Another of Ostrom (1990)'s key principles for enduring common pool institutions is collective choice arrangements. This allows the local community to participate in the decision-making process through village institutions and several committees. Community participation in natural resource management is one of the pillars of CBNRM (Mbaiwa, 2012) and is usually the outcome of sustainable CBNRM. When resource users can derive economic benefits from their resources, they tend to develop positive attitudes to natural resources and therefore use them sustainably, and this promotes participation among them (Gurneya et al., 2016). However, if the benefits are not realised by the local community, they usually become demotivated, and this frequently destroys participation, since the unsustainable use of resources is the outcome. The holistic watershed management in Sukhomojri, India shows that active participation of the local community is needed for the conservation and management of extraction of timber for firewood, brought about by its increased scarcity. It demonstrates that the local community should take an active part in the management of their natural resources (Pathek and Gour-Broone, 1999). The motivation to participate in natural resource management derives from benefits that the resource users receive from the natural resource (UNDP, 2012e). In water resources management in India, the local community in village assemblies is fully involved in determining the management of the watershed, distribution of water, rules about annual repairs as well as penalties for users of unsustainable natural resources Islam and Jain, 2011). Effective participation is also shown in a water case in India where villagers are allowed to make decisions on the management of their water resource through village institutions; several committees are democratically formed in the area. Participation of the local community in the St Lucia water case is sustained by encouragement from the management of the responsible use of natural resources, as well as direct and indirect monetary incentives. Awareness and demonstrations offered to the local community in the study area are important, too (St Jacques, 2009). Often, local community participation in the case studies is low. In Tanzania, Uganda, Nepal and Kenya local participation is also low in the cases examined (Emerton and Mfunda, 1999; Kellert et al., 2000). However, in Botswana, the situation is different, as local community participation is observed (Mbaiwa, 2012). Active participation of the local community in natural resource management is an important element of a successful CBNRM initiative. The sustainability of natural resources is therefore guaranteed if the local community is actively involved in the management of the resources through various committees and village institutions where they are free to make decisions on matters related to their natural resources.

Fisher's Exact Test analysis of the cross-tabulation of the variables further indicates that for a CBNRM initiative to be successful, all eight variables are important. Of the 21 cases analysed, 11 are successful in their CBNRM programmes, five are partially successful while the last five are not successful (*Table 2*). This is because the Fisher's Exact Test P-value in all the variables analysed was .00, which was less than .5, indicating a significant relationship between each variable and the success of the CBNRM initiative (*Appendix 1*). This shows that all the variables assessed in the review, are all important for CBNRM initiative to be successful.

Conclusion

This study analysed CBNRM initiatives in 21 case studies from different countries based on the application of Ostrom (1990)'s eight design principles of common pool regimes to the management of natural resources. The various aspects of the 21 case studies were examined and grouped into the sustainability of natural resources, social institutions sustainability and livelihood sustainability. To ascertain the sustainability of the CBNRM programme in each of the case studies mentioned above, all three areas were examined. All three aspects of sustainability are evident in the successful cases. Most of the dimensions of social institutions' sustainability are achieved by the case studies, such as equity, participation, sense of community ownership, social coherence and encouraging diversity in the community. Livelihood sustainability is also achieved by the cases that achieved a high scoring. Dimensions of livelihood sustainability that were examined include economic and indirect benefits, for example in the areas of education and health, where infrastructure was built to ensure that the community would satisfy its basic needs so as to foster good quality of life in the communities. Natural resource sustainability was achieved in a few cases, as there seemed to be a challenge in balancing the social and natural aspects of sustainability. However, cases from North America seem to have no problem with that, as evidenced by the study. Natural resource sustainability is a major problem in cases from Africa and Asia. Dimensions examined include monitoring and proof of biodiversity protection. Cases that did not score high in the social sustainability and livelihood sustainability dimensions seem to have problems in protecting their natural resources. A successful CBNRM programme is possible when the resource users are motivated to take care of their resources. When the resource users are not motivated, the result is the unsustainable use of resources, which leads to failure in the CBNRM initiative. It is therefore important to promote the social and livelihood aspects of sustainability, such as equity, participation, rights and empowerment, collective choice and conflict mechanisms, to have a successful CBNRM programme. It is recommended that further studies be conducted with larger sample sizes utilising different techniques for increasing the understanding of multiple factors that are essential for achieving effective and sustainable CBNRM initiatives.

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APPENDIX

***Appendix 1.** Cross-tabulation analysis of variables*

CBNRM * Equity

Cross-tabulation

			Equity		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within equity	100.0%	0.0%	23.8%
	Partially Achieved	Count	0_a	5_a	5
		% within CBNRM	0.0%	100.0%	100.0%
		% within equity	0.0%	31.3%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within equity	0.0%	68.8%	52.4%
Total		Count	5	16	21
		% within CBNRM	23.8%	76.2%	100.0%
		% within equity	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of equity categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	21.000[a]	2	.000	.000		
Likelihood Ratio	23.053	2	.000	.000		
Fisher's Exact Test	17.008			.000		
Linear-by-Linear Association	15.188[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.19.
b. The standardised statistic is 3.897.

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*CBNRM * Empowerment*

Cross-tabulation

			Empowerment		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within empowerment	50.0%	0.0%	23.8%
	Partially Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within empowerment	50.0%	0.0%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within empowerment	0.0%	100.0%	52.4%
Total		Count	10	11	21
		% within CBNRM	47.6%	52.4%	100.0%
		% within empowerment	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of empowerment categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	21.000[a]	2	.000	.000		
Likelihood Ratio	29.065	2	.000	.000		
Fisher's Exact Test	22.076			.000		
Linear-by-Linear Association	16.500[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 4 cells (66.7%) have expected count less than 5. The minimum expected count is 2.38.
b. The standardised statistic is 4.062.

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*CBNRM * Participation*

Cross-tabulation

			Participation		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within participation	100.0%	0.0%	23.8%
	Partially Achieved	Count	0_a	5_a	5
		% within CBNRM	0.0%	100.0%	100.0%
		% within participation	0.0%	31.3%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within participation	0.0%	68.8%	52.4%
Total		Count	5	16	21
		% within CBNRM	23.8%	76.2%	100.0%
		% within Participation	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of participation categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	21.000[a]	2	.000	.000		
Likelihood Ratio	23.053	2	.000	.000		
Fisher's Exact Test	17.008			.000		
Linear-by-Linear Association	15.188[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.19.
b. The standardised statistic is 3.897.

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*CBNRM * Monitoring/biological diversity*

Cross-tabulation

			Monitoring / Biological diversity		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0 %
		% within monitoring/ biological diversity	83.3%	0.0%	23.8%
	Partially Achieved	Count	1_a	4_a	5
		% within CBNRM	20.0%	80.0%	100.0 %
		% within monitoring/ biological diversity	16.7%	26.7%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0 %
		% within monitoring/ biological diversity	0.0%	73.3%	52.4%
Total		Count	6	15	21
		% within CBNRM	28.6%	71.4%	100.0 %
		% within monitoring/ biological diversity	100.0%	100.0%	100.0 %

Each subscript letter denotes a subset of monitoring/biological diversity categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	17.080[a]	2	.000	.000		
Likelihood Ratio	20.123	2	.000	.000		
Fisher's Exact Test	15.515			.000		
Linear-by-Linear Association	14.727[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.43.
b. The standardised statistic is 3.838.

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CBNRM * Conflict resolution

Cross-tabulation

			Conflict resolution		Total
			Absent	**Present**	
CBNRM	**Not Achieved**	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within conflict resolution	83.3%	0.0%	23.8%
	Partially Achieved	Count	1_a	4_a	5
		% within CBNRM	20.0%	80.0%	100.0%
		% within conflict resolution	16.7%	26.7%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within conflict resolution	0.0%	73.3%	52.4%
Total		Count	6	15	21
		% within CBNRM	28.6%	71.4%	100.0%
		% within conflict resolution	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of conflict resolution categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	17.080[a]	2	.000	.000		
Likelihood Ratio	20.123	2	.000	.000		
Fisher's Exact Test	15.515			.000		
Linear-by-Linear Association	14.727[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.43.
b. The standardised statistic is 3.838.

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*CBNRM * Collective choice*

Cross-tabulation

			Collective choice		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within collective choice	83.3%	0.0%	23.8%
	Partially Achieved	Count	1_a	4_a	5
		% within CBNRM	20.0%	80.0%	100.0%
		% within collective choice	16.7%	26.7%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within collective choice	0.0%	73.3%	52.4%
Total		Count	6	15	21
		% within CBNRM	28.6%	71.4%	100.0%
		% within collective choice	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of collective choice categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	17.080[a]	2	.000	.000		
Likelihood Ratio	20.123	2	.000	.000		
Fisher's Exact Test	15.515			.000		
Linear-by-Linear Association	14.727[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.43.
b. The standardised statistic is 3.838.

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CBNRM * Local condition rules

Cross-tabulation

			Local condition rules		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within local condition rules	83.3%	0.0%	23.8%
	Partially Achieved	Count	1_a	4_a	5
		% within CBNRM	20.0%	80.0%	100.0%
		% within local condition rules	16.7%	26.7%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within local condition rules	0.0%	73.3%	52.4%
Total		Count	6	15	21
		% within CBNRM	28.6%	71.4%	100.0%
		% within local condition rules	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of local condition rules categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	17.080[a]	2	.000	.000		
Likelihood Ratio	20.123	2	.000	.000		
Fisher's Exact Test	15.515			.000		
Linear-by-Linear Association	14.727[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.43
b. The standardised statistic is 3.838.

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*CBNRM * Sanction activities*

Cross-tabulation

			Sanction activities		Total
			Absent	Present	
CBNRM	Not Achieved	Count	5_a	0_b	5
		% within CBNRM	100.0%	0.0%	100.0%
		% within sanction activities	83.3%	0.0%	23.8%
	Partially Achieved	Count	1_a	4_a	5
		% within CBNRM	20.0%	80.0%	100.0%
		% within sanction activities	16.7%	26.7%	23.8%
	Achieved	Count	0_a	11_b	11
		% within CBNRM	0.0%	100.0%	100.0%
		% within sanction activities	0.0%	73.3%	52.4%
Total		Count	6	15	21
		% within CBNRM	28.6%	71.4%	100.0%
		% within sanction activities	100.0%	100.0%	100.0%

Each subscript letter denotes a subset of sanction activities categories whose column proportions do not differ significantly from each other at the .05 level.

Chi-Square Tests

	Value	Df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	17.080[a]	2	.000	.000		
Likelihood Ratio	20.123	2	.000	.000		
Fisher's Exact Test	15.515			.000		
Linear-by-Linear Association	14.727[b]	1	.000	.000	.000	.000
N of Valid Cases	21					

a. 5 cells (83.3%) have expected count less than 5. The minimum expected count is 1.43.
b. The standardised statistic is 3.838.

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THE EFFECTS OF RURAL DOMESTIC SEWAGE RECLAIMED WATER DRIP IRRIGATION ON CHARACTERISTICS OF RHIZOSPHERE SOIL

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Abstract. With the shortage of water resource being more and more serious day by day, reclaimed water irrigation is an effective approach to solve this problem. In this article, field experiment is conducted to research and analyze the effects of different proportions of rural domestic sewage reclaimed water drip irrigation on characteristics of rhizosphere soil, using groundwater drip irrigation as sample comparison. The results show that: As for soil physical and chemical characteristics, the soluble salt concentration (EC value) of soil solution under reclaimed water drip irrigation is higher than that under groundwater drip irrigation. However, the pH values of soil solution under both conditions are similar. As for soil nutrients, compared with sample comparison, reclaimed water irrigation increases the amounts of organic matter in soil and available phosphorus in soil layer, but has no effect on the contents of available nitrogen and available k. The higher the concentration of reclaimed water in irrigation water is, the more obvious the effect on soil respiration is. Under the condition of drip irrigation, with proper agronomic measure and irrigation system, the adoption of certain proportion of rural domestic sewage reclaimed water irrigation is an effective way to reduce reclaimed water pollution.
Keywords: *soil characteristics; EC value; nutrients*

Intrduction

Reclaimed water resource is a kind of recycled water source, which has great potential value. In China, we are short of water source, especially agricultural water source (Yadav et al., 2002; Ramirez et al., 2002; Lu et al., 2016a). In order to relieve the present situation of water shortage, reclaiming rural domestic sewage and using it for agricultural irrigation is an important way to solve the problem of agricultural water shortage. The effects of reclaimed water on soil physical features change according to the soil characteristics and they mainly show in N, P and salinity contents. The contents of other elements in reclaimed water are similar to that of tap water (Smol et al., 2015; Lu et al., 2016b; Cui and Ouyang, 2015). It has been proved that heavy metals and other organic matters in reclaimed water do not pollute soil obviously. Waste water irrigation, which contains lots of nutrients, helps to increase soil fertility. Since there are traces of saline matters in reclaimed sewage, long-term irrational irrigation can affect soil permeability, for example, excessive salinity

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may accumulate at plant roots, which will change soil components and cause soil to harden; some ions in salinity are poisonous, which can cause physical environment changes; the increase of sodium can reduce soil porosity, which decreases soil's capacity to keep nutrient elements (Lu et al., 2015; Wiśniewska-Kadżajan et al., 2017).The effects of both fertility and pollution of reclaimed water determine that effects of sewage irrigation on physical and chemical characteristics especially the fertile of soil is a hot research topic in reclaimed water irrigation. However, the complexity of migration and transmission of irrigation water source in soil system makes the effects of reclaimed water on the fertile of soil a much difficult topic.

Drip irrigation is an effective way to reduce the pollution of reclaimed water. Drip irrigation is a modern irrigation method that allows water and fluid fertilizer to drip in small flow, long time, and high frequency to the soil with crops roots, on the basis of water and fertilizer requirement law of the crops (Badruzzaman et al., 2013; Rao et al., 2017). It can save increase production, save water, fertilizer, labor and energy and adopt well to different kinds of landform and soil, which helps to improve utilization efficiency of water and nutrients and reduce agricultural non-point source pollution while ensures high yield and grade (Jennings and Ma, 2008; Lu et al., 2016c; Lu et al., 2017). The severe control irrigation time and amount and soil moisture area in drip irrigation allows it to adjust soil moisture and nutrients according to the physical property, crop root system distribution and crop water consumption. Recycling rural domestic sewage by drip irrigation can improve the utilization efficiency of N, P and other nutrients in reclaimed water, save fertilizer and water, improve production and reduce pollutants that might be put into environmental ecosystem. This is significant for relieving water shortage crisis, controlling agricultural non-point source pollution and promoting circular economy development.

Reusing sewage, applying and promoting reclaimed water is an effective way to increase income and reduce expenditure, important support to construct the economic society, an important measure of harmonious development of realizing economic benefits, society benefits and environment benefits (Becerra-Castro et al., 2015; Islam et al., 2015; Lu et al., 2016d; Lu and Shang, 2017). By using reclaimed water, we can save regular water source, keep and replenish groundwater, and relieve the conflict between water supply and demand in countries, which brings remarkable economy benefits. This article researches and compares effects of rural domestic sewage reclaimed water and groundwater drip irrigation on the characteristics of crop rhizosphere soil, with other conditions being the same (Dragović et al., 2014; Lu et al., 2016b). In this article, field experiment is conducted, and "Agricultural surface pollution control integrated demonstration in the south to North Water Diversion Project", in the southern mountain area, Maojian district, Shiyan city, Hubei province, is used as testing area. By measuring related indexes of harvest soil, it shows that recycling rural domestic sewage by drip irrigation can improve the utilization efficiency of N, P and other nutrients in reclaimed water, save fertilizer and water, improve production and reduce pollutants that may be put to environmental ecosystem. It is significant for relieving water shortage crisis, controlling agricultural non-point source pollution and promoting circular economy development. With other conditions same, by comparing the pH value and EC value of rural domestic sewage reclaimed water irrigation (Frantz et al., 2012), this experiment analyzes the effects of nutrients contents in soil on soil respiration, and researches and compares the effects of rural domestic sewage reclaimed water and groundwater drip irrigation on characteristics of crop rhizosphere soil. It provides evidences for evaluation of reclaimed water irrigation effects on soil environment and making secure control method.

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Material and method

Experimental field situation

The experimental area is "Agricultural surface pollution control integrated demonstration in the south to North Water Diversion Project", which is established by Institute of Geographical Sciences and Natural Resource Research, Chinese Academy of Sciences and The Policy and Technology Research Center of South-north Water Diversion Project Office of the State Council. This area is located in the southern mountain area, Maojian district, Shiyan city, Hubei province in the subtropical monsoon climate area. The four seasons there are distinct, and winter period is long while spring is short. In spring, the temperature rises rapidly. In autumn there are lots of rains. In winter, there are few rains and snows and it is not very cold. The annual amount of solar radiation is 106.6 Kcal/cm^2, physiological radiation is 50.4 Kcal/cm^2, and average annual sunshine duration is 1925.8 hours. Annual average temperature is 15.3 °C, with extreme low temperature -14.9 °Cand extreme high temperature 41 °C. The annual accumulated temperature (≥10°C) is 4936.5 °C. There are 246 frost-free days a year. Multi-year average precipitation is 855mm and the precipitations in different year are very different. The precipitation of flood season (from May, 1st to Dec. 20th) takes up about 58%-62% of the whole year, with features of great intensity, short duration and limited infiltration capacity, which can easily scour and erode soil surface. The experimental soil in research is yellow-brown soil and the volume weight of it is 1.56 -1.71g/cm^3.

The reclaimed water used in test station is from the village, in Maojian southern mountain area, Shiyan city, Hubei Province, which is secondary treated reclaimed water. The water quality is stable and the water is just get when used.

Experimental arrangement

The experiment is conducted from May to September, 2011, the growing season of spinach. The cultivation method of spinach is ridge planting: ridge shoulder width: 40cm; center space of two ridges: 100cm; ridge height: 10cm, two lines of plants per ridge; planting space: 20cm. They are irrigated by 5 kinds of irrigation water, labeled as following: T1 (full reclaimed water), T2 (joint irrigation of reclaimed water and groundwater with reclaimed water taking up 70%), T3 (joint irrigation of reclaimed water and groundwater with reclaimed water taking up 50%), T4 (joint irrigation of reclaimed water and groundwater with reclaimed water taking up 20%) and C (full groundwater), as shown in *Table 1*. There are three small areas for each treatment, with 3 ridges of 4m long for each small area, that is 4 m×3 m for each small area. The irrigation method is gravity drip irrigation. Drip tapes are placed at the center of each ridge, the spacing of each dripper is 20cm, same distance as row spacing, dripper discharge is 0.6 L/h, and that is to say each plant has a dripper near root to provide water. Each treatment (including three small areas) is provided with water by a barrel (volume: 180 L). The barrel is put about 2.0 m high from ground. In the second small test area of each treatment, install a set of suction gauge. When the suction gauge shows that soil water potential is under -25 kPa, the plant will be irrigated, 5mm every time, 7 times during growth period, 35mm in total.

Before planting spinach, the field is fertilized with DAP fertilizer, 20kg per mu. During experiment, the area with treatment of reclaimed water irrigation (T1, T2, T3 and T4) will not be fertilized again. But for the area with treatment of full groundwater irrigation (C), 100g urea is dissolved in the water of the irrigation barrel before irrigation, and then the area will be fertilized with it.

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Determination indexes and methods

At the end of spinach growing season, use soil auger to get soil samples under drippers. The sample depth is 0-30 cm and 30-60 cm. The samples from the three areas with same treatment are mixed as one soil sample. After being dried naturally and triturated, the soil is sorted by 1mm sieve. Take exact 20.0g soil, add 100mm deionized-distilled water, sufficiently oscillate, shake up and filter, take supernatant liquid, under 25 ℃, use “DDS-307 type” conductivity meter to measure EC value and meanwhile use "pHS-2C type" meter to measure EC value. Available nitrogen amount in soil is measured by alkaline hydrolysis diffusion method; Available phosphorus amount is measured by 0.05 mol·L-1 $NaHCO_3$ method; Available k amount is measured by NH_4OAc extraction and flare photometer method; Organic matter amount is measured by the potassium dichromate method. Soil respiration is measured by soil partitioning respiration of Li-6400-09 Portable Photosynthesis System produced by American company Li-cor. The measuring position is under dripper, and the period is three days during growing season , at the time 11:00-12:00, three times repeatedly.

Besides, *Table 2* shows the quality of the following water: experimental reclaimed water, which originates from rural domestic sewage, filtered by multi-layer soil infiltration system, groundwater, domestic sewage and reclaimed water. The effects of multi-layer soil infiltration system on treatment of domestic sewage are not obvious. There are many upper limits of indexes in reclaimed water that exceed the upper limits of country’s irrigation standard (Lu et al., 2016b; Khamisi et al., 2013; Furumai, 2008). Under reclaimed irrigation, soil-plant system will digest and absorb pollutants in reclaimed water. Under the condition of drip irrigation, if the water volume of a single time is less, the leaching loss of pollutants will be less. With effective coordination of reclaimed water quality and irrigation volume, we can ensure the high production of crops and prevent pollutants from leaching into water body.

Table 1. *Treatments of reclaimed water drip irrigation with rural domestic sewage*

No.	Treatment	Irrigation water quality
1	T1	Full reclaimed water
2	T2	70% reclaimed water
3	T3	50% reclaimed water
4	T4	20% reclaimed water
5	C	Full groundwater

Table 2. *Test methods and items of water quality and its comparison with country’s irrigation standard*

Items	Groundwater	Domestic sewage	Reclaimed water	Analysis method	Irrigation water quality Standard (GB5084-2005)-vegetable
COD_{Cr} (mg·L^{-1})	3.21~5.83	450~730	68~112	dichromate method	100
NH_3-N (mg·L^{-})	0.08~0.17	73~108	14~29	Nessler ' s reagent colorimetry	-
turbidity (NTU)	0.4~2	20~113	21~98	turbidity meter	-
SS (mg·L^{-1})	2~10	60~190	30~60	gravimetric methods	60
Temperature/℃	10~20	14~38	14~38	thermometer	-

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pH	7~8	6~9	6~9	glass electrode method	6~9
BOD_5 ($mg \cdot L^{-1}$)	1.2~2.33	179~271	28~47	dilution and seeding method	40
DO ($mg \cdot L^{-1}$)	0.21 ~0.14	2.9~5.7	2.0~4.8	Portable Dissolving Instrument	≥ 0.5
TN ($mg \cdot L^{-1}$)	0.99~2.03	53~79	17~37	ultraviolet spectrophotometry	≤ 30
TP ($mg \cdot L^{-1}$)	0.08~0.22	4.9~10.4	4.1~9.3	ultraviolet spectroscopy	≤ 30

Results

Effects of rural domestic sewage reclaimed water drip irrigation on pH value and EC value of soil

Soil pH value is an index of soil acidity-alkalinity, and it is also an important index of critical value of trace elements in soil. The proper pH value for soil is 7.5~8.4. It is generally acknowledged that reclaimed water irrigation will not cause the increase of soil pHvalue (Yi et al., 2011; Gaydon et al., 2012). In this research, in 0~30cm deep soil layer, the pH value decreases gradually with the increasing proportion of reclaimed water in irrigation water. Compared with C treatment, the reductions of T1-T4 are only 5.4%, 2.3%, 1.9% and 1.5% respectively (refer to *Figure 1*). As for 30~60cm deepsoil layer, the difference of soil pH is not obvious. This indicates that compared with full groundwater irrigation, different proportions of rural domestic sewage reclaimed water drip irrigation does not have great effect on soil pH value.

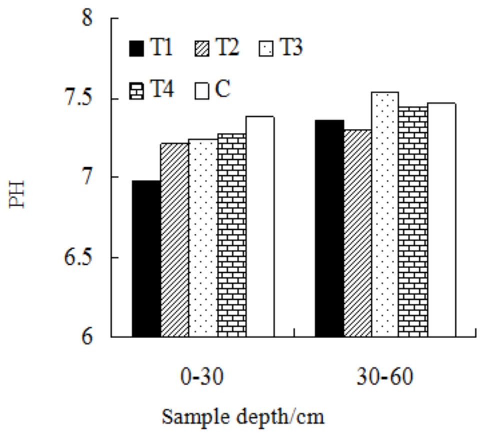

Figure 1. *Effects of rural domestic sewage reclaimed water drip irrigation on soil pH value*

Soil EC value is an index of soluble salt content in soil solution. High concentration of soluble salt will damage plants and cause the death of plant roots. As shown in *Figure 2*, rural domestic sewage reclaimed water irrigation has significant effect on soil EC value. Compared with groundwater drip irrigation, the EC values of upper and lower soil layer under T1 treatment and full reclaimed water drip irrigation are higher. However, the soil EC value under 20% and 50% reclaimed water drip irrigation increases little, especially

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for the lower soil layer, which shows no obvious difference (Darouich et al., 2012; Mouri et al., 2013). In conclusion, high proportion of reclaimed water drip irrigation significantly increases EC value of upper soil layer.

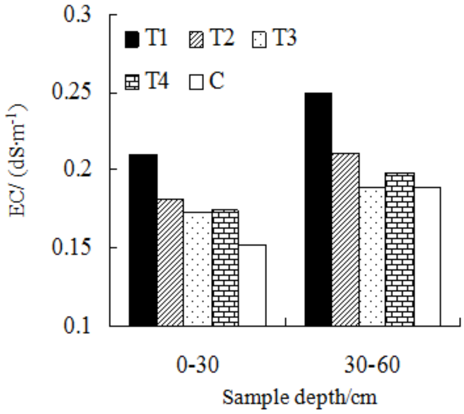

***Figure 2.** Effects of rural domestic sewage reclaimed water drip irrigation on soil EC value*

Effects of rural domestic sewage reclaimed water drip irrigation on soil nutrition content

Soil can provide everlasting and low-dose available nutrients (Nayak and Prasanna, 2007;). A lot of domestic and overseas researches on soil fertility levels after reclaimed water irrigation are made, and one coincident conclusion is made as following: reclaimed water irrigation can significantly improve soil fertility and long-term irrigation can reduce artificial fertilization (Sidhu et al., 2013; Hooper et al., 2014; Samsó and García, 2013).The potassium amount increases more in reclaimed water irrigation area, which is caused by its absorption and transformation in soil. Soil organic matter content is an important index of assessing soil fertility, which is relevant to various kinds of soil nutrients and has significant effect on soil physical property such as water retention and supply capacity, porosity and aggregate degree, etc (Lu et al., 2016a). As shown in *Figure 3*, with the increase of reclaimed water proportion in irrigation water, the organic matter amount increases, and the increasing trend is more obvious in upper layer soil. The organic matter contents in soil under full reclaimed water drip irrigation and 70% reclaimed water drip irrigation are obviously higher than other, which indicates that reclaimed water drip irrigation can increase organic matter contents in soil.

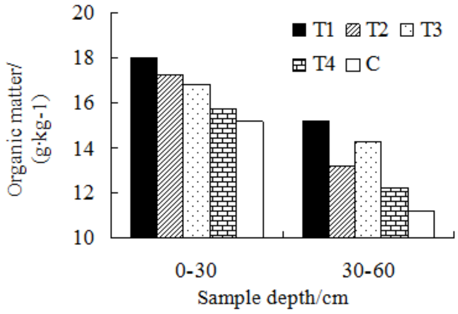

***Figure 3.** Effects of rural domestic sewage reclaimed*

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In upper soil layer of 0~30cm deep, the available nitrogen contents in soil under T1 (full reclaimed water irrigation) and C (additional nitrogen) treatment are much higher than others (refer to *Figure 4*). In 30~60cm deep soil layer, the nitrogen content in soil under T1 treatment is higher, and no obvious difference under other treatments. Generally speaking, except for soil under full reclaimed water irrigation, the increase of available nitrogen is not obvious for soil under different proportion of reclaimed water drip irrigation. The reason may be that reclaimed water drip irrigation increases the organic matter contents which increases the growth of plant and then promotes the consumption of nitrogen in soil, so that there is no obvious difference of nitrogen under T2, T3 and T4 treatments.

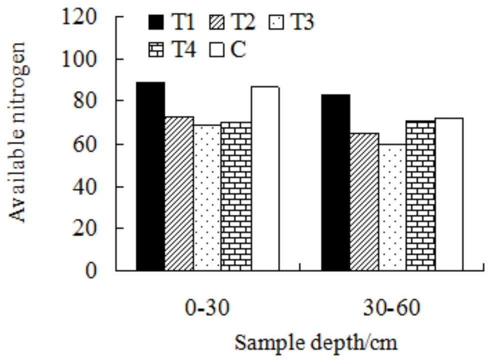

Figure 4. *Effects of rural domestic sewage*

Different proportions of reclaimed water drip irrigation have great effect on available phosphorus contents in spinach rhizosphere soil (refer to *Figure 5*). In 0~30cm deep soil layer, except for soil under T4 treatment (20% reclaimed water drip irrigation), the available phosphorus contents in soil under T1, T2, T3 treatments are higher than soil under full underground water drip irrigation. In lower soil layer (30~60cm), available phosphorus contents in soil under T1 and T2 treatments are obviously higher than other treatments. There is no obvious difference between other three kinds of treatments. It indicates that high proportion of reclaimed water drip irrigation can greatly increase available phosphorus content in soil.

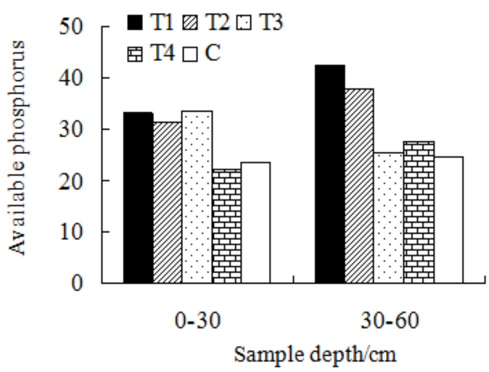

Figure 5. *Effects of rural domestic sewage reclaimed*

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As shown in *Figure 6*, different proportions of reclaimed water drip irrigation have little effect on available K content in spinach rhizosphere soil. In 0~30cm deep soil layer, with increase of reclaimed water proportion in irrigation water, the available K content increases gradually, but the difference is not obvious. In lower soil layer (30~60cm), available K contents in soil with full reclaimed water drip irrigation and 70% reclaimed water drip irrigation are a little higher than full groundwater drip irrigation. There is no obvious difference among other treatments. Generally speaking, rural domestic sewage reclaimed water drip irrigation has little effect on available K contents in spinach rhizosphere soil. Bajaj and Singh (2015) considers that reclaimed water drip irrigation can provide nutrients for soil, especial potassium fertilizer, so that we can use 24．5～97．8kg / hm^2 less potassium fertilizer than regular irrigation.

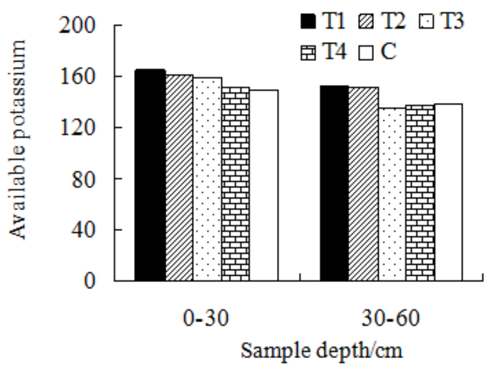

Figure 6. *Effects of rural domestic sewage reclaimed*

From the experimental results, under combined effects of internal and external factors, such as physiological activity of plant root cells, maintenance and management practice, change of climate conditions, etc., if irrigating spinach field by slightly alkaline (pH>7) reclaimed water, the pH value of the soil will decrease a little, which means the acid is enhanced. The enhanced soil acid will accelerate the humification process of organic matter, which will enhance soil acid and accelerate the liberalization and ionization process of N, P, and Ketc. This makes them in an existential state that is easy to run off with water, and digest and use by plant. So that elements, such as N, P, K, etc., will not accumulate in soil and neither will growth failure or large-area death happens because of the accumulation of N, P, K, etc.

Effects of rural domestic sewage reclaimed water drip irrigation on soil respiration

Soil respiration refers to all the metabolic activities in soil that can produce carbon dioxide (Yang et al., 2015; Rezgui et al., 2016). It mainly includes respiration of plant roots (part of autotrophic respiration), heterotrophic respiration of soil microbes and animal, and it is an import index that reflects the soil fertility characteristics. *Figure 7* shows effects of different proportion of reclaimed water drip irrigation on soil respiration. As shown in *Figure 7*, different proportions of reclaimed water drip irrigation can all promote soil respiration. The larger the proportion is, the more obvious the promotion and the faster the respiratory rate is. This is because reclaimed water drip irrigation does not only increase nutrients in soil such as organic matter, but also increases the amount of various kinds of microbes in soil. Microbes in soil use organic matters as energy

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substance, decompose them step by step and release them in form of CO_2 and mineral nutrients, which provides plants with available nutrients.

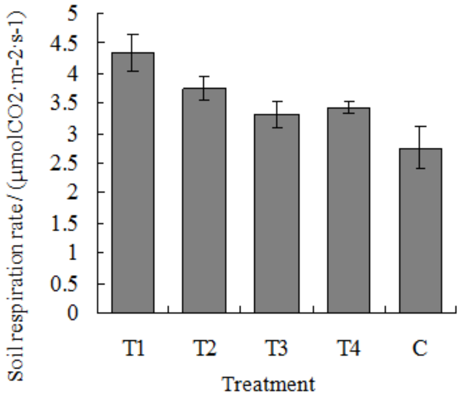

Figure 7. *Effects of rural domestic sewage reclaimed water*

Conclusion

Rural domestic sewage reclaimed water drip irrigation will not cause obvious or anomalous change of soil pH value, but high proportion of reclaimed water drip irrigation can increase EC value of upper soil layer. Relatively speaking, rural domestic sewage reclaimed water drip irrigation has little effect on available nitrogen and available K, but has significant effects on organic matter andavailable phosphorus. Besides, there is no anomalous change in plant growth because of sewage pollutants accumulation in soil; it indicates that reclaimed water, used as irrigation water, can improve soil fertilizer to some extent, and in the aspect of heavy metal, reclaimed water irrigation is safe. Reclaimed water drip irrigation can promote soil respiration. The larger the proportion of reclaimed water is, the faster the respiratory rate is.

Therefore, under the condition of drip irrigation, with proper agronomic measure and irrigation system, certain proportion of rural domestic sewage reclaimed water can be adopted to irrigate crops. The results also show that, to some extent, the pollutants in reclaimed water still accumulate in soil, and they have effect on plant growth. As for the effect degree and period, we need to make long-term experiments and do further observation and research.

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Conflict of Interests. The authors declared that they have no conflict of interests to this work.

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RIVER RUNOFF CHANGES IN THE PAST MILLENNIUM UNDER EXTREME CLIMATIC CONDITIONS IN CHINA

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Abstract. In this study, Poyang Lake catchment with frequent drought and flood disasters in recent years was chosen as the study area. Due to the influence of global climate changes, great changes have taken place in the hydrological and climatic process of Poyang Lake in the past half a century, leading to the increase of extreme weather and hydrological events, shrinkage of water, threat to wetland ecosystem and other problems. First, historical climate data simulated by Atmosphere-Ocean General Circulation Models CCSM4 and ECHAM5 were obtained, which were then applied to Poyang Lake catchment with down-scaling method. The hydrological model WATLAC can be driven by upper climate data along with other data so that we get the runoff series in this catchment for the past one thousand years. Based on the research, it was found that the runoff series were in significant positive correlation with precipitation series. The frequency of extreme runoff was consistent with that of precipitation in both models. The peak value was 0.03, indicating the period was 30 years. The wavelet power spectrum contours of runoff and precipitation series generate significant signals in 20～40-year scale. The signal is the strongest in a scale of about 30 years, which further confirms the runoff of Poyang Lake catchment has a macro cycle of about 30 years.
Keywords: *millennial time scale; climate model; runoff changes; hydrological cycle; Poyang Lake catchment*

Introduction

The Poyang Lake, located in the middle and lower reaches of Yangtze River, is the largest fresh-water lake in China. As one of the few large lakes linking to the river, Poyang Lake plays an important role in water conservation, flood control, climate regulation, ecological protection, etc (Zhu and Zhang, 1997). Due to the influence of global climate change (Guo et al., 2006), great changes have taken place in the hydrological and climatic process of Poyang Lake in the past half a century, leading to the increase of extreme weather and hydrological events (Liu and Ren, 2014), shrinkage of water (Yu and Hu, 2010), threat to wetland ecosystem and other problems (Jiang et al., 2010). Will this situation continue to deteriorate in the future under the influence of climate changes? This paper, based on statistical down-scaling method, thus analyzes

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runoff changes of Poyang Lake in the past millennium under the extreme climatic conditions, aiming to offer scientific guidance for the flood and drought control, ecological protection, development and utilization of water resources and to ensure the coordinated development of the economy and society in this area.

Overview of Research Area

Poyang Lake is located on the south side of the lower Yangtze River. It is the largest lake connected with Yangtze River. The length from south to north of Poyang Lake is 173 km and the average width from east to west is 16.9 km, with the width of the widest part at about 74 km. The narrowest part of Poyang Lake is only about 2.8 km wide. The total length of the lake circle is 1200 km (Jiang et al., 2007). There are five rivers which contribute to Poyang Lake, namely Ganjiang River, Fuhe River, Xinjiang River, Raohe River and Xiuhe River. After regulation and storage, water in Poyang Lake runs into Yangtze River, which is an important characteristic of seasonal influent-effluent lakes. There are significant periodic variations in water levels of Poyang Lake within the year and among different years. In the wet season, the water level is high and the lake surface is large, with the highest lake surface area being 4070 km^2; in dry seasons, the water level is low, with the lowest lake surface area being no more than 150 km^2 and the lake water being contained in the channels. The maximum water surface area ever recorded in the wet season is about 27 times of the minimum ever recorded in the dry season, which makes Poyang Lake a lake in wet seasons and several rivers in dry seasons. Variation of water level is mainly induced by the effects of the five rivers mentioned above and the Yangtze River. The main flood season of the 5 rivers is from April to June, when the water level in Yangtze River is comparatively low and the flood water into Poyang Lake can be discharged into the Yangtze River rapidly and the water level in Poyang Lake can be maintained; from July to September, incoming water volume from the five rivers decreases, but water level in Yangtze River is high, which makes the water level in Poyang Lake high; after the end of September, water level in Yangtze River decreases, dry season of the five rivers is coming and the water level in Poyang Lake gradually becomes lower and lower. According to statistical data, the water level variation of Poyang Lake within a year is up to 10 m, which is unique in large lakes (Xu and Chen, 2009).

Research Methodologies

Climate Model

Climate model usually refers to general circulation model (GCM), a mathematical-physical model of the earth system, which determines the characteristics of the Earth's atmosphere based on mathematical equations formed in dynamic, physical, chemical and biological process of the earth system. The climate model used to simulate changes of the climate system, the response time and process, and the spatial features is a key method to study climate changes on seasonal and interdecadal scale. Climate model involves the simple energy-balance equation and the complex earth system model. Currently the earth system model (ESMs) couples atmosphere, ocean, land and sea ice, with some part of the model even coupling aerosol, carbon cycle, dynamic vegetation and atmospheric chemical process (Liu and Zhang, 2009). Atmosphere-ocean general

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circulation model (AOGCMs) couples the dynamic changes of atmosphere, ocean, land, ice and snow in the climate system. The Coupled Model Intercomparison Project Phase 5（CMIP5） develops a research plan to simulate climate in the past millennium. More than 20 climate model organizations drive the climate field through the same indicators like solar radiation, volcanic ash, greenhouse gases and etc. And results of near-surface temperature, upper atmosphere and atmospheric circulation characteristics are calculated through physical model like dynamics, heat, and water volume of climate model (Liu, 2011). The USA general climate model: The Community Climate System Model 4 (CCSM4) and European Centre Hamburg Model (ECHAM5) are adopted in this paper to simulate climate (Qin et al., 2014).

The CCSM4 and ECHAM5 are used in the paper as hydrological drive, for they can better simulate the climate in East Asia (Yu and Harrison, 1996). CCSM4 climate model developed by National Center for Atmospheric Research is well received in China (Blackmon et al., 2001). Recently, Tian et al. (2012), Tian and Jiang (2013) have proved that CCSM4 model can better simulate climate features of East Asia and China. Thus it is suitable for long-time climate model researches. The ECHAM5 model proposed by the Max Planck Institute for Meteorology improves the dynamic coupling function of atmosphere, ocean, land and ice and snow, based on ECHAM4 model. The improved model has better simulation (Yu et al., 2009). In recent years, Bueh and Lin (2003) have used ECHAM4 to simulate East Asian monsoon circulation and predict its future changes. Zhai et al. (2009) use ECHAM5 to forecast distribution of drought and flood in China before the year 2050. These researches show that the two models can better simulate regional climate system in China.

So far, most coupled models (AOGCMs) have a relatively low spatial resolution. It is therefore difficult to make reasonable predictions about climate changes on regional scale. The down-scaling methods then come into being, and have since been widely applied to overcome AOGCM's weakness. Statistical down-scaling method is a powerful tool to obtain small-scale climate data from large-scale climate data. Firstly, empirical relationship between large-scale climate factors (predictors) and regional climate factors (predictors) should be established. Then the relationship is used as the output of global climate model or regional climate model and corresponding information of any place can be obtained (Fan et al., 2005).The paper gets related information of Poyang Lake based on the simulation of CCSM4 and ECHAM5 within the framework of IPCC-CMIP5. And monthly temperature and rainfall series of Poyang Lake in the past millennium are obtained through down-scaling methods. Yu et al. (2013) use historical literature and facts from archaeology study to verify the simulation results. The verification shows that GAMs' simulation of rainfall is acceptable. Modern climate data are obtained from monthly temperature and rainfall records of 17 stations of National Secondary Meteorological Station between the year 1951 and 2015, as well as the station positions.

Hydrological Data

Hydrological model: A Water Flow Model for Lake Catchements (WATLAC) is a mathematical model that simulates surface water and underground water of lake catchment. Unlike most hydrological models with a general summary of underground runoff, WATLAC is improved in this aspect and can better simulate the hydraulic relationship between lakes and inland rivers (Zhang, 2007). Driven by rainfall and evaporation, the model involves two simulation processes: surface runoff and

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underground runoff. It takes into consideration major hydraulic process, such as canopy interception, catchment evapotranspiration, soil water storage, slope runoff, groundwater recharge and runoff, and river runoff. Surface runoff is simulated by spatial discrete raster (Zhang, 2009). Each raster has different hydrogeological parameters and input data by remote sensing interpretation. In view of different characteristics of saturated soil and unsaturated soil, for underground runoff, finite difference is used to disperse saturated zone and analytic equation is used to simulate unsaturated soil (Liu and Zhang, 2009).

Input data like Digital Elevation Model (DEM), land use, evapotranspiration, soil characteristics, daily rainfall, and a large quantity of information including soil moisture, soil and underground water recharge, and lake runoff can be outputted. The main structure of the model is shown in *Figure 1*.

Researches on Poyang Lake based on WATLAC model have been recognized all over the world. For example, Fan et al. (2005) verify the model based on measured data between 1960 and 1989 of Waizhou Station and Xiajiang Station. During the calibration period, the annual runoff of the two stations has an error less than 8%. This shows that the model has better simulation. Ye et al. (2011) combine ECHAM5 and WATLAC to predict the runoff of Poyang Lake in the context of future climate. Compared with the measured data of six hydraulic stations, the predicted result has a certainty factor above 0.7, indicating a better simulation of the model. Li et al. (2014) use measured data of lake runoff, lake level, outlet volume and etc. to verify the model. The certainty factor is above 0.75, showing a better simulation of the model. These researches show that distributed hydrological model WATLAC has been successfully applied in the research of Poyang Lake. The paper therefore, adopts the model to establish runoff series of Poyang Lake in the past millennium.

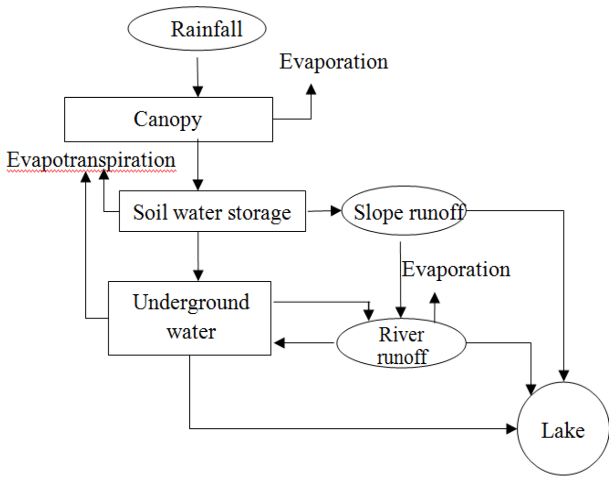

Figure 1. *The simulated hydrology process of Model WATLAC*

Result Analysis

Features of Rainfall-Runoff Changes

This paper focuses on the features of interannual-interdecadal changes based on an understanding of rainfall-runoff series in the past millennium obtained from the mean value of rainfall-runoff at different time scale of 1 year, 5-year, 10-year, 20-year, 30-

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year. Simulation results of rainfall series based on CCSM4 and ECHAM5 are shown in *Figure 2*. *Figure 2* respectively shows simulating changes of 10-year average anomaly for depth of rainfall runoff of Poyang Lake in the past millennium based on CCSM4 and ECHAM5. CCSM4 model includes the runoff series between 1040 and 1850, 810 years in all. ECHAM5 model includes the runoff series between 1000 and 1959, 960 years in all. The two models differ a little in the simulation of dry-wet changes during the MWP and LIA periods.

For the MWP period, CCSM4 model shows that runoff ranges around the mean value and that dry and wet situation appear alternatively. In terms of the rainfall, 45% of the years have a dry weather, and 38% have runoff. The distribution is relatively even. For the LIA period, dry-wet situation changes drastically and easily causes extreme drought or flood. 53% of the years have rainfall and 41% have runoff. Both appear in the dry years. The wet years appear alternatively with dry years. The dry-wet situation changes significantly. On the whole, according to CCSM4 model, dry and wet situation appear alternatively and the changes of rainfall and runoff are similar. For the MWP period, ECHAM5 shows that 56% of the years have a low rainfall and 49% have a low runoff. For the LIA period, 59% of the years have a low rainfall and 55% have a low runoff. According to this model, dry-wet situation remains for a long time.

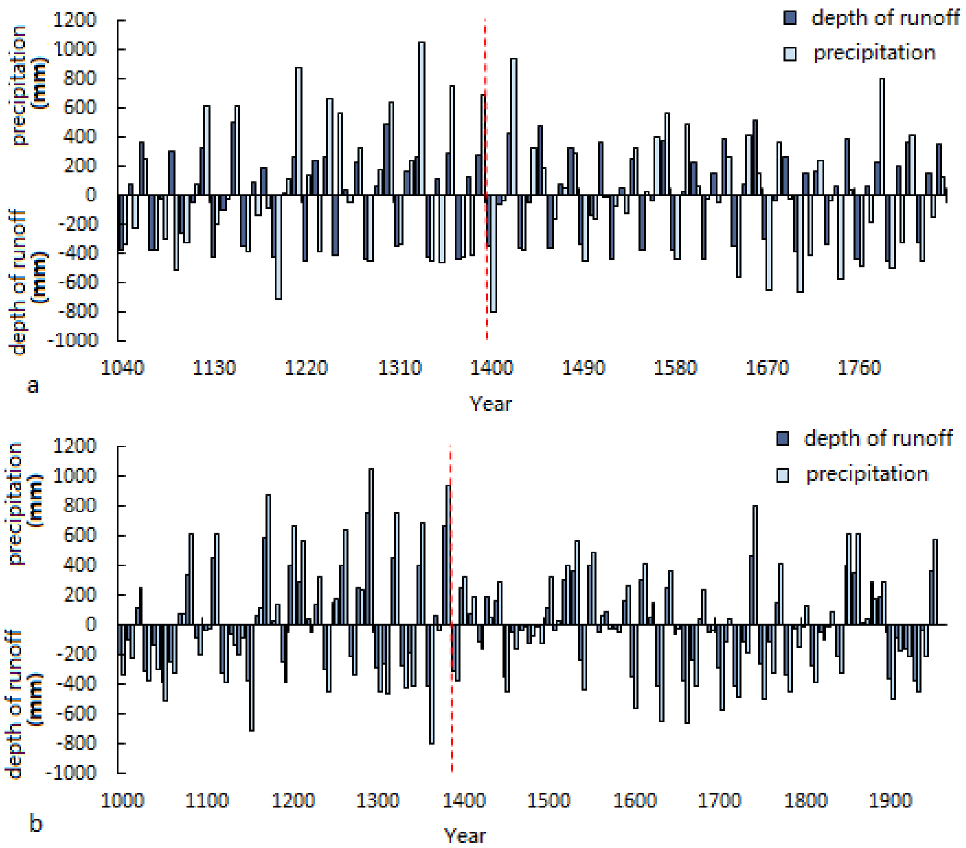

Figure 2. *Variances of anomaly of precipitation and runoff on 30-year time scale. Picture (a) represents the model CCSM4 and (b) is ECHAM5. The red dash line divided the picture into period MWP and period LIA*

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Periodic Changes of Rainfall-Runoff

The periodical changes of rainfall-runoff depth series based on CCSM4 and ECHAM5 are shown in *Figure 3*. Fast Fourier Transform (FFT) results can easily identify strong and weak changes of time series on different scales. *Figure a* and *b* in *Figure 3* show that both models have several peak values, indicating the strongest vibration at certain frequency. Corresponding frequency of rainfall-runoff depth series at the peak value has a higher matching level. This indicates that rainfall and depth of runoff have the same vibration period on the time scale. In CCSM4 model frequency above confidence curve involves 0.033, 0.101 and 0.133. In ECHAM5 model frequency above confidence curve involves 0.034, 0.066 and 0.133. The recurrence intervals of rainfall-runoff series in the two models are similar, suggesting the synchronous changes of runoff process and rainfall process. In view of the statistical meaning of the period, here listed are the first three periods.

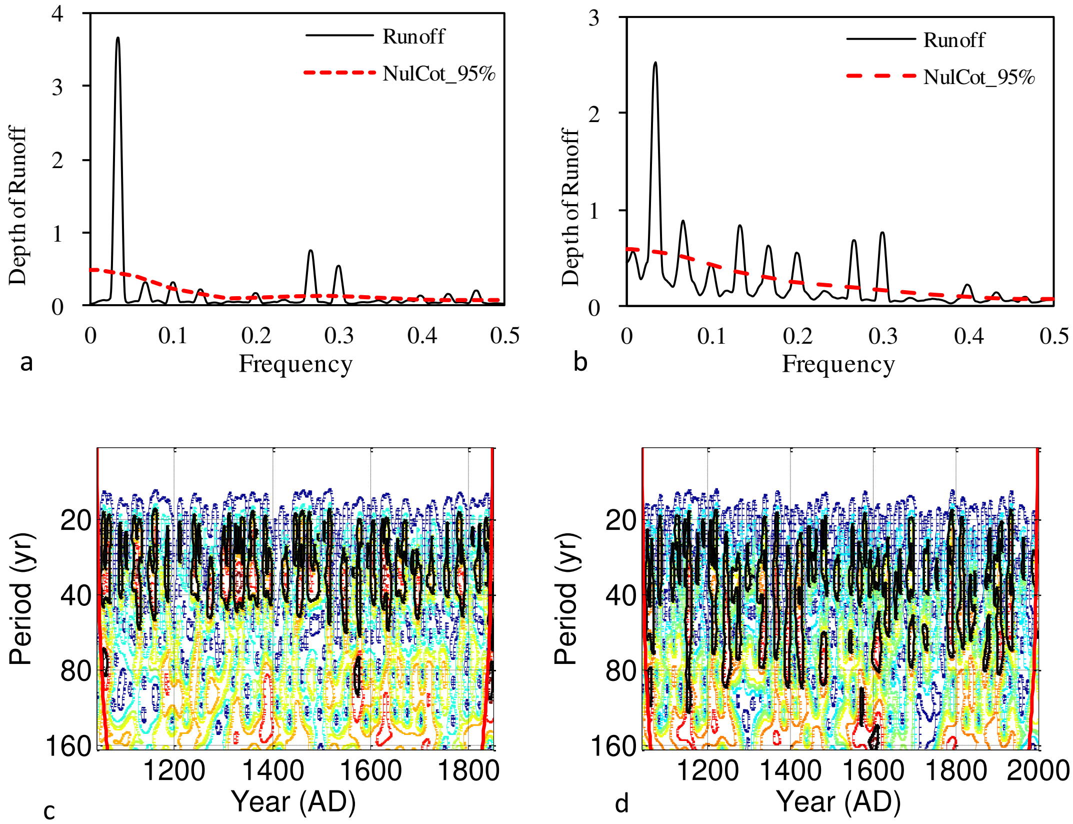

Figure 3. *(a and b) Fast Fourier transform spectral power spectrum of depth of runoff. The black full line and dash line indicate spectral power of precipitation and depth of runoff respectively. And the red ones mean their own 95% confidence line. (c and d) Wavelet power spectrum contours of depth of runoff for CCSM4 model and ECHAM5. It was plotted by time series against periods, in which the 95% confidence contour was in black part and the red full line meant cone of influence. Any part below the red line is not reliable.*

Period distribution in Wavelet Transform module square time frequency distribution contour is similar to that in FFT. *Figure 3c* shows that in CCSM4 model both rainfall and depth of runoff are strongest on time scale of 20 to 40-year, with the energy center

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around 30-year. According to *Figure 3*, in ECHAM5 model confidence region is at 20 to 80-year, but the energy center with continuous changes is basically around 30-year. Power spectrum contours of rainfall and runoff series in both models are basically the same. This further shows that though flow process is influenced by some factors, it mainly changes with rainfall process on time scale. According to the analysis results of the models, the period of rainfall-runoff change of Poyang Lake in the past millennium is around 30-year.

Hydrological and Meteorological Series and Frequency and Period of Extreme Events

Figure 4a and *4b* are FFT power spectrums of rainfall based on CCSM4 and ECHAM5 respectively. For the sake of comparison, *Figure 4c* and *4d* show FFT power spectrum of rainfall-runoff respectively based on the two models.

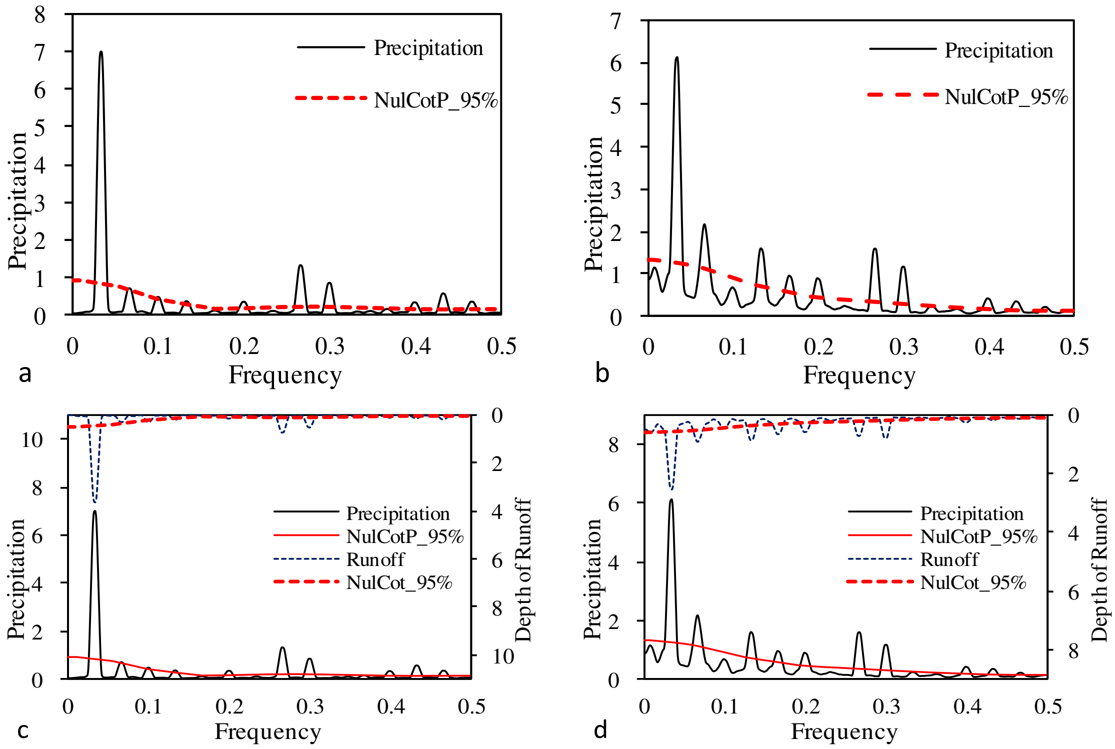

Figure 4. *(a and b) Fast Fourier transform spectral power spectrum of precipitation and depth of runoff. (c and d) Spectral power spectrum between precipitation and depth of runoff. The black full line and dash line indicate spectral power of precipitation and depth of runoff respectively. And the red ones mean their own 95% confidence line*

According to *Figure 4*, power spectrum change distribution of rainfall series and runoff series are basically the same. The peak value of rainfall series is bigger, indicating a high frequency of extreme rainfall events. Similar power spectra reflect the corresponding relationship between the extreme runoff and the climate.

Figure 5a is Wavelet Transform module square time frequency distribution of rainfall anomaly based on CCSM4 model. *Figure 5b* is Wavelet Transform module square time frequency distribution of rainfall anomaly based on ECHAM5 model.

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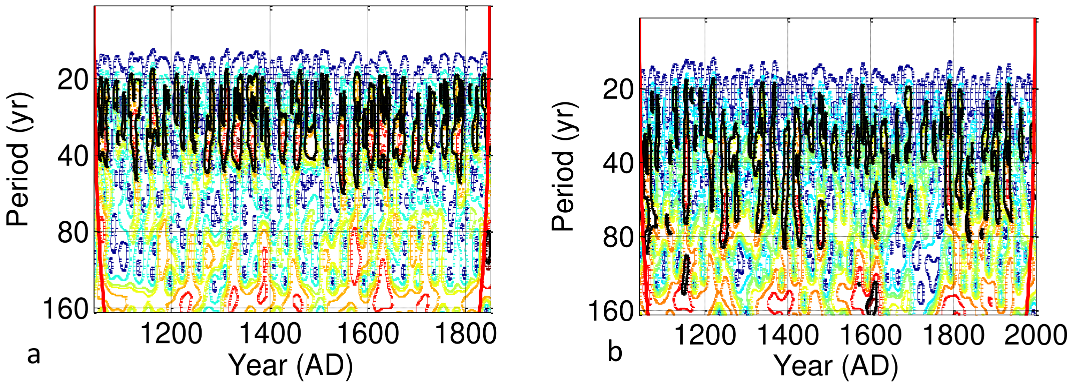

Figure 5. *(a and b) Wavelet Transform module square time frequency distribution of depth of precipitation for CCSM4 model and ECHAM5 model. It was plotted by time series against periods, in which the 95% confidence contour was in black part and the red full line meant cone of influence. Any part below the red line is not reliable*

Rainfall period shown in *Figure 5* is similar to that in FFT, and to module square distribution of runoff depth in *Figure 3*. In CCSM4 model rainfall has the strongest energy between 20-40 year, with the energy center at around 30-year. .ECHAM5 model has confidence region between 20 and 80-year, but the energy center with continuous changes basically remains at 30-year. According to the figure, both models show that in the past millennium Poyang Lake had similar rainfall process and runoff process, with 30-year being the period. This again displays the corresponding relationship between the runoff and the climate.

Conclusion

This paper simulates the runoff series of Poyang Lake in the past millennium and explains the hydrological changes, features and dynamic mechanism of this area under the influences of climate changes. Nowadays extreme hydrological events happening in Poyang Lake area are mainly caused by rainfall and surface confluence. And a regression analysis of historical situation can offer reliable scientific proof for predicting future extreme hydrological events. The runoff period of Poyang Lake in the past millennium is 30 years, similar to the current runoff research results of Poyang Lake. This further proves the influence of climate changes on hydrological process in the area on the time scale.

- Rainfall and runoff of Poyang Lake in the past millennium have a similar variation trend based on CCSM4 and ECHAM5 models through down-scaling methods. This shows the basic characteristics of atmosphere circulation under the influence of radiation and the formation of basic rainfall pattern. Runoff changes a little differently based on the two models. In ECHAM5 model there is a longer time of dry years, while in CCSM4 model dry years and wet years appear alternatively. In both models, dry years in LIA period is more than those in MWP period.
- According to the FFT analysis of runoff series based on CCSM4 and ECHAM5 models, the runoff series shows main period 30 years and 29 years with peak value, secondary period between 10 and 15 years, and the 3rd period around 7 years. The analysis shows that the main period is related to atmospheric

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oscillation and Pacific decadal oscillation; the secondary period is related to solar activity; and the 3rd period is related to El Nino influenced by radiation and atmospheric action. Changes of atmospheric oscillation reflect the macro features of a regional climate. In terms of changes on interdecadal and interannual scale, what matters is interior vibration of climate system and air-sea-land interaction. According to the wavelet analysis, relatively strong signals appear between 20 and 40 years in CCSM4 model and between 20 and 80 years in ECHAM5 model, but the peak value of energy appears around 30 years. The results are similar to FFT results. Within the same model, module square distribution of rainfall-runoff series has a similar energy distribution. This indicates that rainfall and runoff have the same vibration changes on the millennium scale. This phenomenon reveals the atmospheric oscillation in the millennium, which is helpful in understanding features and rules of regional climate changes against the natural climate changes.

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THE COMPOSITION AND STRUCTURAL FEATURE OF PLANT COMMUNITY IN DIFFERENT KARST STONY DESERTIFICATION AREAS

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Abstract: Information on the species composition and community structure of vegetation at different successional stages is useful for understanding successional mechanisms and for selecting species for vegetation recovery. We investigated the composition and structure of plant community in different succession stages by setting 270 sample plots in all 5 types stony desertification areas of Guizhou province, China. The results show that: (1) in the early succession stage of karst rocky desertification area, the environment adaptable species mainly are pioneer species of light-demanding and drought-tolerant. With the succession, species change following a variation trend of "increasing-decreasing-increasing", Shrub stage had the largest number of plant species. (2) There is difference in plant composition in different succession stages of 5 types stony desertification areas. (3) During natural succession stages, the cover degree, predominance degree, and density of plant community represents the horizontal structural feature of karst stony desertification area. The difference in predominance degree and density is considerable, and in certain proportion relation. (4) In early-mid succession stages of plant community, plants are in contagious distribution, while in climax community succession stage, most plants are in random distribution and less are in contagious distribution. From above results, it can be concluded that the variation feature of plant communication in karst stony desertification areas follows certain natural succession law, therefore, species collocation and optimal configuration should be arranged according to natural succession law for control of stony desertification.
Keywords: *community succession; life form; type of stony desertification; plant arrangement*

Introduction

The total carbonatite outcrop area of China is about 1,300,000 km^2, which is distributed in most provinces of China. China's southern region (including Guangxi, Yunnan, Sichuan, Chongqing, Hubei and Hunan provinces based on Guizhou as center) has carbonatite outcrop area of over 540,000 km^2, which is featured by the largest outcrop area in 3 linked karst regions, the strongest karst development, the most landscape types, and the most complex ecological environment (Yang et al., 2014; Zhang et al., 2014). Moreover, the aforesaid southern region is the major area where subtropical karst forest locate. Being different from foreign karst regions, the southern karst region of China is mainly composed of hard carbonate rock before triassic period,

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in which the limestone porosity in all geologic ages is lower than 2%, dolomite porosity is generally lower than 4% with compressive strength under 1000kpa/cm^2 (Jiang et al., 2014). All these characteristics exert great influence to karst development. With the substantial rise of Cenozoic quaternary tectonic movement, the karst topography of carbonate development in all geologic ages becomes more comprehensive, which is distributed in different altitudes, forming the karst landform with world's longest time span and best spatial continuity. Moreover, the karst forest grown in this area has its own particularity in global range, therefore, the ecological study on karst forest in this area is of more important scientific value (Wen et al., 2015).

In the fragile karst ecologic environment, human irrational social economic activities cause prominent human-earth system conflict, vegetation deterioration, water and soil loss, bare rock, land productivity decline or even loss, making ground surface manifested in desert landscape. Such evolution process and outcome is called stony desertification (Jiang et al., 2014). In karst region, stony desertification brings severe impact on the survival, production, and life of rural people, and seriously hinder the sustainable socioeconomic development of this area. However, stony desertification is an inherent and zonal landscape in karst region. For example, the development of karst forest vegetation in Maolan of Guizhou province, which is a primordial forest vegetation, provides references for the recovery of forest vegetation in karst stony desertification area. It is of great significance for stony desertification control to use water, soil, gas, heat in a balance and skillful way in such harsh environment and figure out the succession law and interspecific competitions of plant community in karst region (Liu et al., 2011). Therefore, the species composition and spatial structure of plant community in natural succession stages in karst region should be clearly understood, which provides theoretical basis for species selection in early stage and optimization allocation of stony desertification control.

Profile of Research Area

Located in eastern part of the Yunnan-Guizhou Plateau, Guizhou province of China has an area of 176,167km^2 including mountainous region and hilly land accounting for 92.5% of total province area, which is an inland plateau mountainous area with karst development, crosses of mountains and rivers, abundant resources, various nationalities and no plains (*Fig. 1*). The karst outcrop area of Guizhou province is 109,084.58km^2, accounting for 61.92% of total province area. In 2011, the stony desertification area of Guizhou province is about 30,200 km^2, accounting for 17.16% of total province area. The rock combination characteristics of Guizhou province can be categorized into 3 major combination types including homogenous carbonate rock combination, combination of carbonate rock intercalated with clastic rock, and interbedded combination of carbonate rock and clastic rock. The homogenous carbonate rock combination can be further divided into homogenous limestone, homogenous dolomite, and mixed combination of limestone and dolomite. The homogenous limestone is mainly distributed in the south, northwest, southwest of Guizhou province, with area 30, 676km2, accounting for 17.4% of total province area. The homogenous dolomite is mainly developed in the northeast of Guizhou province, with area of 22,990km2, accounting for 13.1% of total province area (Tan, 2006).

Research areas were selected in Libo county, Luodian county, Puding county, Wudang district, Xiuwen county, Pan county, Jinsha county, Bijie county, Weining

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county, Sinan county, Yanhe county, Kaili city, Tongren city, Xingyi city, Zhenfeng county (Huajiang valley). The profile of research areas is introduced in *Table 1*, including place name, longitude and latitude, lithology, stony desertification area, and percentage of stony desertification area to total county (city, district) area.

Table 1. *Profile of research areas*

Region(county, city)	Percentage of stony desertification area km^2/total county (city) area	Altitude (m)	Longitude and latitude	Name of small sampling site	Lithology distribution
Libo county	658.71/27.08	742	N25°15′41.5″ E108°03′54.9″	Maolan	limestone
Luodian county	1085.26/36.06	684	N25°32′16.9″ E106°38′41.5″	Zhangjiakou, Bangeng of Yongjin village	limestone
Puding county	390.93/35.82	1330	N27°22′19.1″ E105°44′49.2″	Houshan, Miaopuhoushan, Sankuaitian of Caojin village	limestone
Wudang district	183.03/19.02	1081	N26°42′24.3″ E106°53′26.7″	Xiaba	dolomite
Xiuwen county	250.1/23.25	1228	N26 °48′46.8″ E106°32′50.3″	Long chang	dolomite
Pan county	1008.85/24.88	2050	N25°39′10.6″ E104°22′0.4″	Wangjia grave of Guanping town	limestone
Jinsha county	616.22/24.38	1156	N27°25′16.4″ E106°14′25.4″	Nongjia cave of Meng village, Yangliu of Chengguan town	limestone
Bijie county	902.53/26.45	1558	N27°15′13.7″ E105°19′55.8″	Stone bridge of Yachi town	limestone
Weining county	1103.91/17.53	2430	N27°07′7.5″ E103°54′8.4″	Liqi rock of YiNa town, Xiaojianshan of Jinzhong town	limestone
Sinan county	344.34/15.4	655	N27°52′43.8″ E108°08′20.8″	Dongkou of Donghua village, Yangjiashan of Xujiaba village	dolomite
Yanhe county	711.16/28.8	793	N28°31′11.2″ E108°24′43.0″	Black water village (Nanbutuo, Chaoyang village), Guanzhou town (Zhenbian, Qingshan temple), Siqu town	dolomite

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Tongren city	256.48/17.54	796	N27°34′26.0″ E105°6′44.0″	Plum blossom reservoir of Chadian town, Niuchangpo of Yutang village	dolomite limestone
Kaili city	250/19.15	756	N26°31′12.0″ E107°52′42.6″	Yatang, Baiyang	dolomite
Xingyi city	1062.94/36.52	1195	N25°07′22.0″ E104°56′37.4″	Wanfenglin scenic entrance of Bajie town, Yingpanshan in Pogang nature reserve of Zhengtun town	limestone
Zhenfeng county	576.68/38.14	565	N25°39′57.1″ E105°40′16.7″	Banwei, Dingtan	limestone

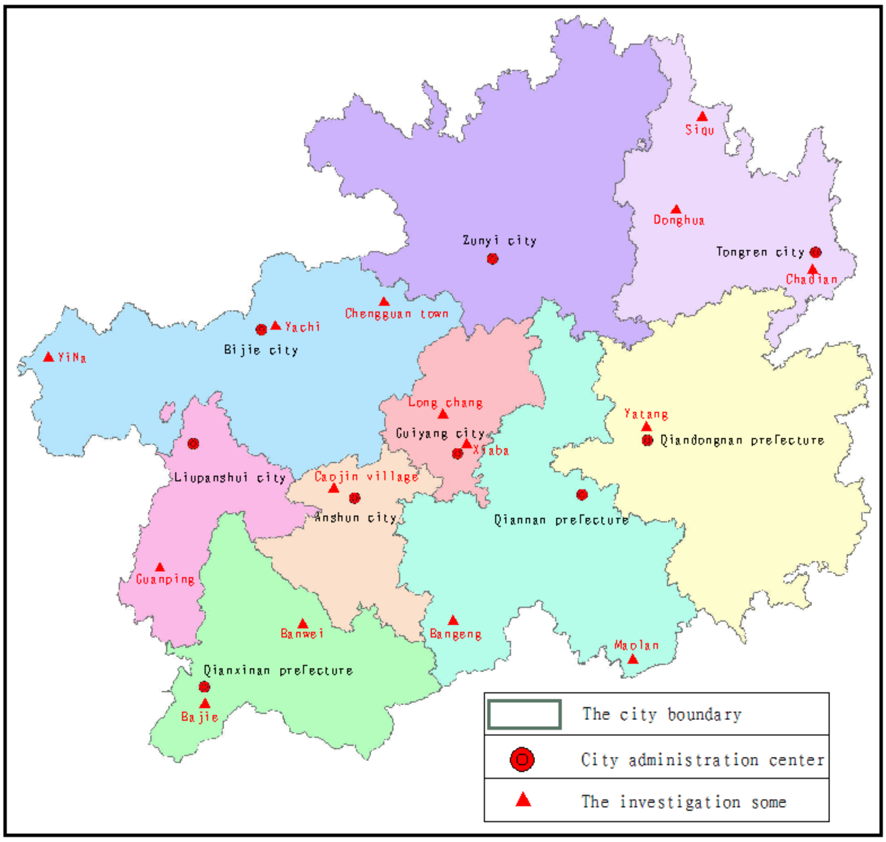

Figure 1. *Distribution of research areas in Guizhou province*

Research Method

Sample-plot survey of plant community

Ecosystem evolution is a long-term course. A natural recovery from degraded grass slope back to climax evergreen and deciduous broad-leaved mixed forest will takes tens or even hundreds of years (Yu, 1998). By setting 270 sample plots in 5 desertification

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areas of Guizhou province, this paper presents investigation on phytocoenology of plant community in different succession stages (Yu et al., 2011) [herbosa succession stage (I), shrub-grass community succession stage(II), brush-shrub community succession stage (III), shrub community succession stage (IV), tree-shrub community succession stage (V), high-forest community succession stage or climax community stage (VI), investigations were conducted 3 times for each stage] using method of substituting spatial sequence for time sequence (Muellerdombois and Ellenberg, 1974). The sample plot areas can be $20m^2$, $100m^2$, $100m^2$, $100m^2$, $160m^2$, $400m^2$. In each square sample plot, there arranges a 2m×2m shrub layer quadrat and a 1m×1m herb layer quadrat. Record the species, quantity, DBH (diameter at breast height), ground diameter, height, crown diameter, cover degree of arbors, shrubs, young trees in each quadrat, and judge whether each plant seeds or sprouts. The species, abundance, height, and cover degree of *herbaceous* plant were investigated as well.

Annotation: According to the outline of rocky desertification in karst region comprehensive treatment planning (2006y to 2015y), China will be divided into eight desertification control type area, among them, there are five rocky desertification in Guizhou province.

Determination of plant life forms

Life form: According to life-form system proposed by Raunkiaer, terrestrial plant can be divided into 5 life forms by taking growing height and location of dormant bud and renewal bud in bad seasons as division criterion.

Phanerophyte (Ph): the dormant bud is at least 25cm high above the group, which can be divided into 4 sub-forms according to plant height:

Macrophanerophyte (Ma): height>30m, for instance megaphanerophyte.

Mesophanerophyte (Me): height 8-30m, for instance microphanerophyte.

Microphanerophyte(Mi): height 2-8m, including microphanerophyte and shrub.

Nanophanerophyte (N): 2m>height>25cm, including shrub and undershrub.

Chamaephyte (Ch): the renewal bud locates above soil surface while under 25cm, mainly include subshrub *herbaceous* plants.

Hemicryptophyte (H): the renewal bud locates in soil layer, which will be partially or fully dead on winter ground, mainly including renascent herb.

Cryptophyte (Cr): the renewal bud locates deeply in soil or water, mainly including aquatic renascent herb plant in bulbous, tuberous or rhizomatic types

Therophyte (T): overwintering as a seed. In this paper, phanerophyte was further divided into Evergreen plant (E) and Deciduous plant, (D). For the convenience of distinguishing, Epiphytic plant (Ep) is listed separately in category system

Division method of adapted species

Community succession is a process where dominant community species changing from one to another. In karst area with complex habitat, there is not one but multiple species that can be adapted to the habitat during the same succession stage (Zhu, 2003). According to the variation of dominant position of main compositional species of deteriorated community during natural recovery process, the adaption level of species can be classified. Specific methods are shown as below:

Based on population as entity, degree of dominance (i.e. important value of population) as attribute, dominance matrix of population during community restoration was established, which means establishing 141×421 original data matrix using quantity index of 421 plant species in141 quadrats.

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$$y = \begin{bmatrix} x_{11} & x_{12} & x_{13} & x_{14} & x_{15} & x_{16} & x_{17} & x_{1j} \\ x_{21} & x_{22} & x_{23} & x_{24} & x_{25} & x_{26} & x_{27} & x_{2j} \\ x_{31} & x_{32} & x_{33} & x_{34} & x_{35} & x_{36} & x_{37} & x_{3j} \\ x_{41} & x_{42} & x_{43} & x_{44} & x_{45} & x_{46} & x_{47} & x_{4j} \\ x_{51} & x_{52} & x_{53} & x_{54} & x_{55} & x_{56} & x_{57} & x_{5j} \\ x_{61} & x_{62} & x_{63} & x_{64} & x_{65} & x_{66} & x_{67} & x_{6j} \\ x_{71} & x_{72} & x_{73} & x_{74} & x_{75} & x_{76} & x_{77} & x_{7j} \\ x_{i1} & x_{i2} & x_{i3} & x_{i4} & x_{i5} & x_{i6} & x_{i7} & x_{ij} \end{bmatrix} = \begin{bmatrix} x_{11} & & x_{1j} \\ & & \\ x_{i1} & & x_{ij} \end{bmatrix} \quad \text{(Eq. 1)}$$

Where i represents sample plot, j represents species, x_{ij} represents dominance (important value) of the j th species in the i th quadrat.

After establishing dominance matrix, DCA (Detrended Correspondence Analysis) was adopted for sorting using eigenvector modified based on CA/RA. The primary shaft was divided by DCA into a series of intervals, in which the mean value was set as zero to adjust the coordinate value of the second shaft. This method was effective method for vegetation analysis, which overcame the arch effect of CA/RA, improved sorting accuracy, and realized the results that were the most consistent with Gaussian community model (Zhang, 2004). After that, CANOCO4.5 analysis software was used for analysis and drawing (Zhang et al., 2000). The results aggregated together were merged into a group.

Study on species diversity

Species diversity was measured using Shannon-wiener diversity index (D):

$$D = 3.3219\left[\lg N - \frac{1}{N}\left(\sum n_i \lg n_i\right)\right] \quad \text{(Eq. 2)}$$

community dominance was measured using Simpson index (C):

$$C = \sum n_i \frac{(n_i - 1)}{N(N-1)} \quad \text{(Eq. 3)}$$

Uniformity was measured by uniform index based on Shannon-Wiene diversity index (J):

$$J = \frac{\lg N - \frac{1}{N}\left(\sum n_i \lg n_i\right)}{\left[\lg N - \frac{\alpha(S-\beta)\lg\alpha + \beta(\alpha+1)\lg(\alpha+1)}{N}\right]} \quad \text{(Eq. 4)}$$

Where N is the number of species individuals; n_i is the number of individuals of the ith species; S is the number of species, βremainder after dividing N by S, αis (N-β)/S (ShaoLin, 1996).

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Heterogeneity of community space

According to geostatistics research method, set 30m×30m quadrats on main sample plots of herb community, shrub community, high-forest community, and climax community, and then partition quardrats into 1m×1m adjacent grids. The habitat variables and heterogeneity in each grid was investigated, including mean soil thickness of various microhabitat types (stone-drain, swallet, stone cavern, stone cistern, soil surface, stone surface), as well as mean soil thickness, soil moisture, litter depth and litter coverage in each quadrat. In addition, collect soil sample from each grid, test the pH value of soil sample, and draw biotope distribution map by measuring scale of 1:500. The investigation was finished at August to September of 2009. The habitat index of soil capacity was proposed in this research, of which the definition is the actual soil volume that can be held in habitat. The soil capacity of each quadrat is the sum of soil capacities of all microhabitats. The soil capacity of each microhabitat is balanced by the product of mean soil thickness and microhabitat distribution area. The distribution area of microhabitat was calculated by paper weighing method according to the distribution map. Soil moisture was measured in field using TDR300 soil-moisture content analyzer, and the soil pH value was measured in door using PHS-3C acidometer in October, 2009.

As different constants normally have different units and variation degrees, which makes it difficult for practical variable explanation. Therefore, after standardization of variable data by standard deviation, the geostatisticcal common software GS+7.0 was used for variogram analysis, Moran's I analysis, and fractal dimension analysis.

Results and Analysis

Species composition of plant community in karst stony desertification area during natural succession stage

Species composition is a necessary element of biotic community. Interspecific coordinated development is related to community succession. The species quantity and composition are very important indexes for evaluating the species diversity, and also the foundation for the stable development of community and ecosystem.

With the succession of community, the species of herb commmunity gradually decrease during recovery stages, while slowly increases in succession stage of high-forest, with generally a V-shape variation tendency. In the early recovery stage of community when the environment condition is harsh, species that can be adapted to harsh conditions are mainly light drought-enduring pioneer species, including *Pteridium revolutum*, *Imperata cylindrica* var. major, *Senecio scandens*, *Trifolium*, *Miscanthus si nensis Anderss*, *Bidens pilosa*, *Erigeron annuus*, *Arthraxon hispidus.* With the change of environment, there are considerable light drought-enduring woody pioneer species migrating here and settling down. In climax community, succession stage, many light Annual herb species are eliminated by the competition, while some shade-tolerant herb plants are more adapted to such environment, developing into stable climax community and settling down, such as Through investigation, the types of species of herb community in peak-cluster depression of stony desertification area are always in leading position during all recovery stages, wherein the highest value is 76, while the lowest value is 56. In plateau, trough valley, canyon, fault basin of stony desertification area, there types of species of herb community can be as much as 75, 61, 63, 56, and as less as 37, 31, 34 and 23, respectively (See *Fig. 2*).

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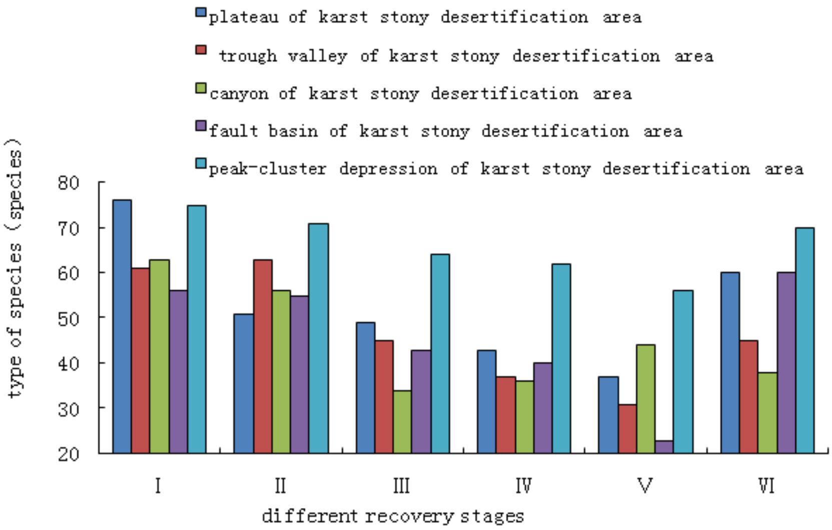

Figure 2. *Variation of species of herb plants in karst stony desertification area during different recovery stages*

With the succession of community, the species of woody plant shows a variation tendency of "increasing-decreasing-increasing". This is mainly because that in the early recovery stage, the species are less, while with the change of environment, the species of woody plant start to increase and reach saturation in shrub stage. After that due to the limitation of vegetation space, some species are eliminated by competition. In tree-shrub transition stage where species competition is the most significant, the early stage of arbor-shrub-grass is also formed, and many species are eliminated because of inadaptation to the transition from light role to shade-tolerance role. In high-forest succession stage, the arbor-shrub-grass layer has been fully formed, shade-tolerance species migrate in and settle down, leading to the increase of species.

According to statistic data, in plateau, trough valley, canyon, fault basin, and peak-cluster depression of karst stony desertification area, the species of woody plant can be as much as 118, 113, 77, 42, 161, respectively, wherein the type of species of wood plant in fault basin is relatively less, which is 42. However, the least number of types of wood plant species in plateau, trough valley, canyon, fault basin, and peak-cluster depression of karst stony desertification are 20, 26, 25, 17, 36, respectively (*Fig. 3*).

The change law of the entire community is consistent with that of woody plant communities (*Fig. 4*). The more the type of species is, the more complex the species composition is, the more stable the ecosystem is, and the stronger the resistance to disease and pest is. Therefore, there are diversity of species during disposition of species, including the *rosaceous* plants, *rutaceous* plants, and *hamamelidaceae* plants in early stage as well as *fagaceae* plant, canella, *magnoliaceae* plants, *theaceae* plants in late stage. In the early vegetation recovery stage, it should focus on species selection during artificial disposition of species, while in the mid-late stage, it should focus on species conserving during structural adjustment.

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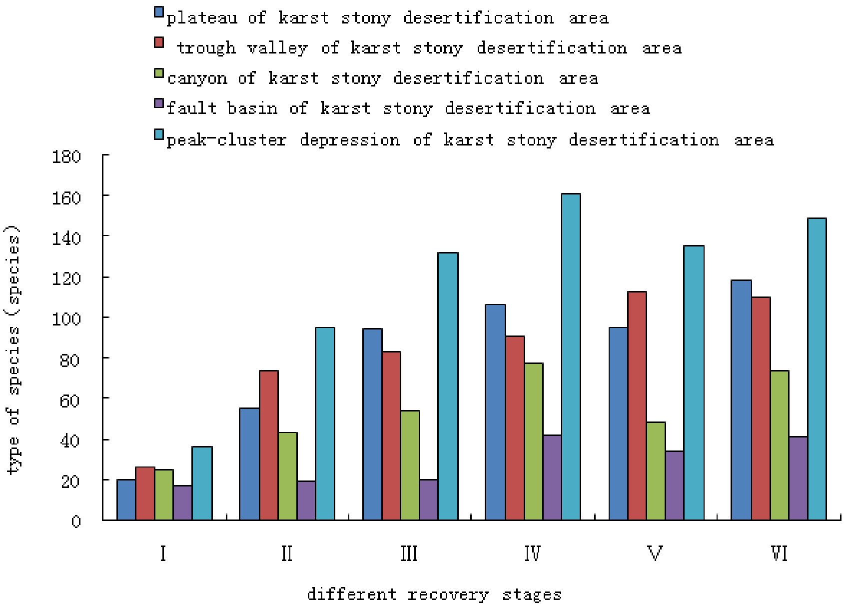

***Figure 3.** variation of species number of woody plant in stony desertification area during different recovery stages*

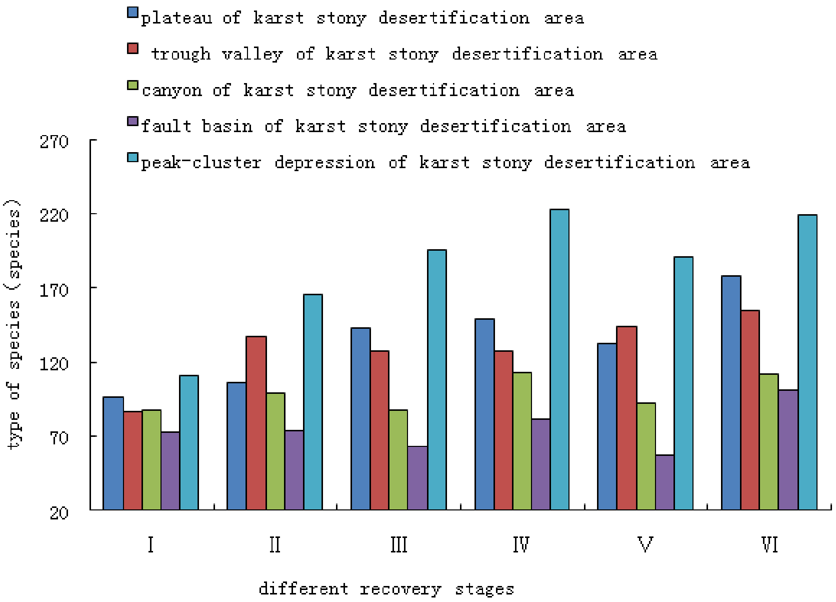

***Figure 4.** variation of species number of community in stony desertification area during different recovery stages*

Life-form composition of plant community in karst stony desertification area during natural succession stage

Life-form plants at different stony desertification areas include therophyte (T), cryptophyte (Cr),hemicryptophyte (H), chamaephyte (Ch), nanophanerophyte Ph(N), microphanerophytes Ph(Mi), mesophanerophyte Ph(Me), megaphanerophyte Ph(Ma), Vine plants (EP), evergreen plant (E), deciduous plant (D). However, there is difference

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in life form composition ratio at different stony desertification areas during different succession stages.

In the early succession stage, woody plants are dominant species. With the succession of community, woody plants gradually reduce, and then start to increase by a slight rate in climax community stage. This is because that in the early succession stage where the environment condition is harsh, there is lack of water and severe drought, species that can be adapted to such environment condition are mainly plants with short life span, which is overwintering as a seed, followed by dried-up overground part and underground part regeneration. The therophyte herb plant is featured by large quantity, small individual, and short life span, which is adapted to environment by R-strategy and change in large extent. In the mid-late succession stage, most tree and shrub plants migrate hear, while herb plants gradually decrease, and most remained species are shade-tolerant herb plants, such as pilea notata, pteridophyta and Carex plants. With the successions of community, phanerophyte (Ph) gradually increase, which mainly include Ph(N) and Ph(Mi) in early stage as well as Ph(Mi) and Ph(Me) in mid-late stage. The variation tendencies of Ph(N) and Ph(Mi) are consistent, both of which are first increasing and then decrease; the variation tendency of Ph(Me) is basically identical to that of Ph(Ma), both of which are gradually increasing.

In the early succession stage, life form components are mainly *herbaceous* plant, Ph(N), and Ph(Mi), with ratio of 6:3:1; In the middle succession stage, life form components are mainly *herbaceous* plant, Ph(N), and Ph(Mi), with ratio of 3:3:4; In the late succession stage, life form components are mainly *herbaceous* plant, Ph(N), Ph(Mi), and Ph(Me), with ratio of 2:3:3:2 (See *Table 2*).

Table 2. *Life form composition ratio of community at different stony desertification areas at early, mid, and late stage of natural succession*

Stony desertification area	Early stage		Middle stage		Late stage	
Plateau of karst stony desertification area	C:Ph(N):Ph(Mi)	6:3:1	C:Ph(N):Ph(Mi)	3:3:4	C:Ph(N):Ph(Mi):Ph(Me)	2:3:3:2
Trough valley of karst stony desertification area	C:Ph(N):Ph(Mi)	6:2:2	C:Ph(N):Ph(Mi)	3:3:4	C:Ph(N):Ph(Mi):Ph(Me)	2:3:2:3
Canyon of karst stony desertification area	C:Ph(N):Ph(Mi)	6:3:1	C:Ph(N):Ph(Mi)	3:3:4	C:Ph(N):Ph(Mi):Ph(Me)	3:3:2:2
Fault basin of karst stony desertification area	C:Ph(N):Ph(Mi)	6:3:1	C:Ph(N):Ph(Mi)	3:4:3	C:Ph(N):Ph(Mi):Ph(Me)	2:3:3:2
peak-cluster depression of karst stony desertification area	C:Ph(N):Ph(Mi)	5:3:2	C:Ph(N):Ph(Mi)	3:3:4	C:Ph(N):Ph(Mi):Ph(Me)	2:2:3:3

Note: C represents *herbaceous* plant, Ph(N) represents nanophanerophyte, Ph(Mi) represents microphanerophyte，Ph(Me) is mesophanerophyte.

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Structural feature of plant community at karst stony desertification area during natural succession stages

Due to the influence of topographic relief, illumination, and humidity, different species are normally distributed at different areas with different density and cover degree, leading to the difference in horizontal structure of whole community. This paper mainly studied the horizontal structure feature and variation of plant community at karst stony desertification area during different succession stages in term of cover degree, predominance degree, and density.

Herbaceous layer community gradually reduces from grass slope stage to high-forest stage; shrub layer first increases and then decreases, while tree layer gradually increases from V to VI stage. The cover degree of plant community is insignificantly changed, with small difference and basically identical variation tendency among different species, which is generally over 80%. The *herbaceous* layer, shrub layer, tree layer reach the largest cover degrees of their own when they are at constructive layer.

Although the cover degree of community is merely changed, the cover degrees of herb, shrub, tree layer are significantly changed. The cover degree of herb layer community gradually decreases from 90.6%-79% in grass slope stage to 32-10% in high-forest stage. In bare rock stage, the cover degree of herb community is even lower, which ranges from 5% to 20%; In grass-slope stage, the cover degree of shrub community ranges from 3.9% to 17%; In shrub community succession stage, the cover degree of shrub layer is the highest, which are 81.7%, 80%, 85%, 80% and 82.5% in plateau, trough valley, canyon, fault basin, peak-cluster depression of karst stony desertification area, respectively; the cover degree of high-forest layer is the highest during high-forest succession stage, which are respectively 73.3%, 80%, 80%, 75% and 71.7% in above areas, with very small difference (*Fig. 5*).

The predominance degree of community is mainly manifested by variation of plant thickness accumulation in horizontal direction. The predominance degree of shrub layer community is manifested by variation of plant ground diameter accumulation in horizontal direction, while that of tree layer community is mainly manifested by variation of plant DBH (at breast height) diameter accumulation in horizontal direction.

In trough valley, fault basin, and plateau of karst stony desertification area, the predominance degree of plant community shows variation tendency of "increasing-decreasing"; while in canyon and peak-cluster depression of karst stony desertification area, the variation of predominance degree of plant community is "increasing-decreasing-decreasing". At canyon of karst stony desertification area in high-forest stage, the predominance degree of shrub layer is high, while the predominance degree of tree layer is low, which indicates such area is dominant by small diameter species during shrub succession stage, while is dominant by large diameter species during tree succession stage. At peak-cluster depression of karst stony desertification area, the predominance degrees of shrub layer and tree layer are both high during high-forest recovery stage, which indicates the large diameter species and small diameter species are both in substantial quantity, and both biomass and stand volume per unit area are in high level (*Fig. 6*).

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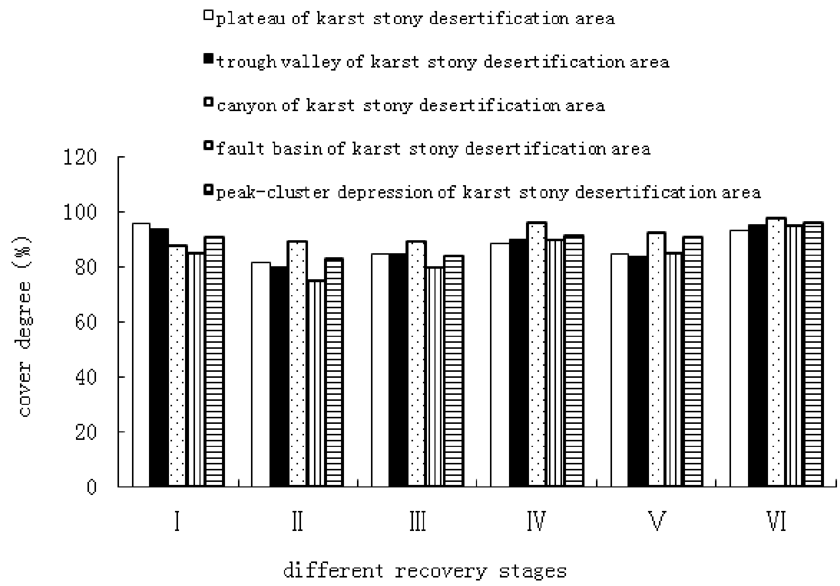

Figure 5. *Cover degree of plant community at karst stony desertification area during different succession stages*

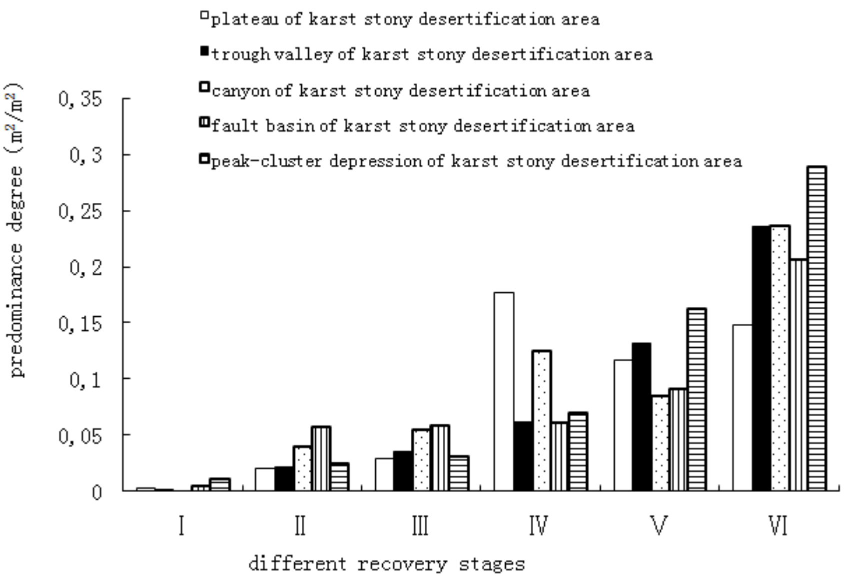

Figure 6. *Predominance degree of plant community at karst stony desertification area during different succession stages*

In early succession stage, the variation range of predominance of plant community are respectively 0.0019-0.02m2/m2, 0.0012-0.021m2/m2, 0.001-0.0396m2/m2, 0.0053-0.569m2/m2, 0.011-0.0253m2/m2 in plateau, trough valley, canyon, fault basin, peak-cluster depression of karst stony desertification area.

The density variation of shrub layer is "increasing-decreasing-increasing" with the succession of community. This is due to that in grass slope stage, herb plant is the dominant species; with the migration of woody plants, the herb plants start to decrease while woody plants gradually increase. After reaching a certain quantity, some species

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will be naturally reduced or eliminated due to interspecific competition of vegetation space, while those with strong competence start to enter into tree layer, use large amount of vegetation space, cover the illumination, making the light plants gradually eliminated due to the inadaptation to shady and cool environment. Therefore, the density of community during tree-shrub transition stage is smaller than that during shrub succession stage. After reaching high-forest succession stage, some shade-tolerant species will migrate in, breed on lower light, and species quantity in shrub layer gradually grow. This is the reason why shrub layer density show variation of "increasing-decreasing-increasing". While the density of tree layer at karst stony desertification area shows an increasing tendency.

The density variation of the whole community is consistent with that of shrub layer, which is "increasing-decreasing-increasing"(*Fig. 7*). This indicates the increase of tree layer is slow, which does not change the variation tendency of the whole community density. Many small shrubs are in aggregated distribution or in clustered distribution, such as *myrsine*, *berberis aggregata*, *rhamnus heterophylla*, chinese *spiraea*, *C.horizontalis perpusillus*. In particular, the *berberis aggregata* in one cluster can be as much as 178 plants. If all plants in one cluster are taken into density, thhen the lowest density of shrub layer in grass-slope stage can account for about 1/4 of the highest density of tree layer in tree succession stage. In addition, there are some sprouting plants including Sichuan hazel, *platycarya longipes*, *Vitex negundo* L., bamboo leaves pepper, and small fruit rose root, which are normally in quantity of several plants or dozens of plants. Plants in clustered distribution which are mainly small shrubs show advantage in density.

In this paper, the number of genet of plants in clustered distribution is regarded as 1. Therefore, in early succession stage, the density of community is relatively smaller, and then gradually increases with the successions, finally reaches the maximum value in the late succession stage. This indicates during community collocation, plant community is in small-density distribution in early succession stage, and then in large-density (including the total density of shrub and tree) distribution in mid-late succession stage. If community structural adjustment is carried out in the late succession stage, it should focus on the density of tree layer, which is 0.48-0.97 plant/m^2, averaging at 0.65 plant/m^2 in the early stage; 2.16-2.97 plant/m^2, averaging at 2.41 plant/m^2 in the middle stage; 0.11-0.42 plant/m^2, averaging at 0.21 plant/m^2 in the late stage (*Table 3*).

Table 3. *Densities of constructive species layer at different karst stony desertification areas in the early, mid, late natural succession stage*

Stony desertification areas	Early stage	Middle stage	Late stage
Plateau of karst stony desertification area	0.56	2.25	0.14
Trough valley of karst stony desertification area	0.95	2.16	0.11
Canyon of karst stony desertification area	0.76	2.27	0.26
Fault basin of karst stony desertification area	0.50	2.97	0.13
Peak-cluster depression of karst stony desertification area	0.48	2.38	0.42

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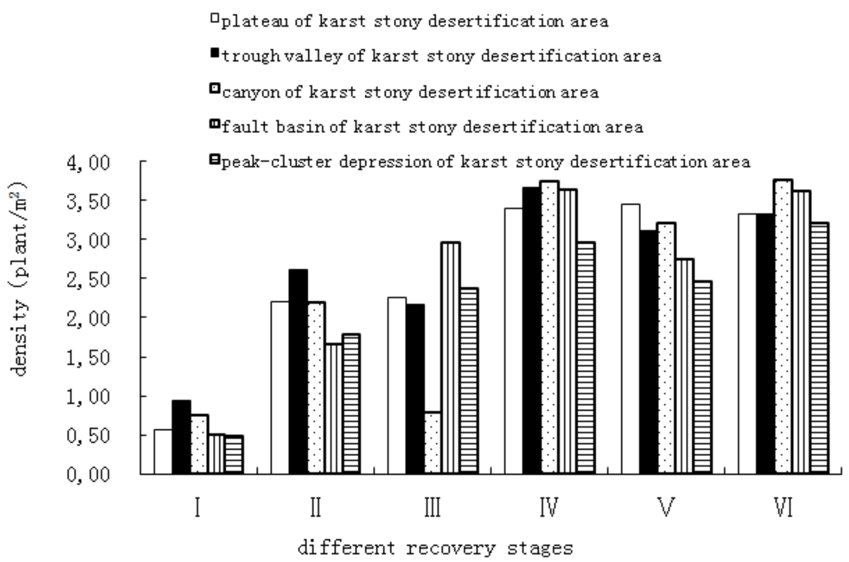

Figure 7. *Community densities in karst stony desertification areas at different recovery stages*

Through investigating the characteristics of spatial distribution of dominant species at different community layers during different succession stages, it can find that the dominant species in all layers are in aggregated distribution in herb and shrub succession stages, while the dominant species in all community layers during high-forest stage is generally in aggregated distribution, but with weaken aggregation intensity and smaller aggregation scale as compared with two former stages, showing variation from aggregated distribution to random distribution in certain degree. In climax succession stage, the dominant species at all community layers is generally in randomly distribution (*Figs. 8* and *9*).

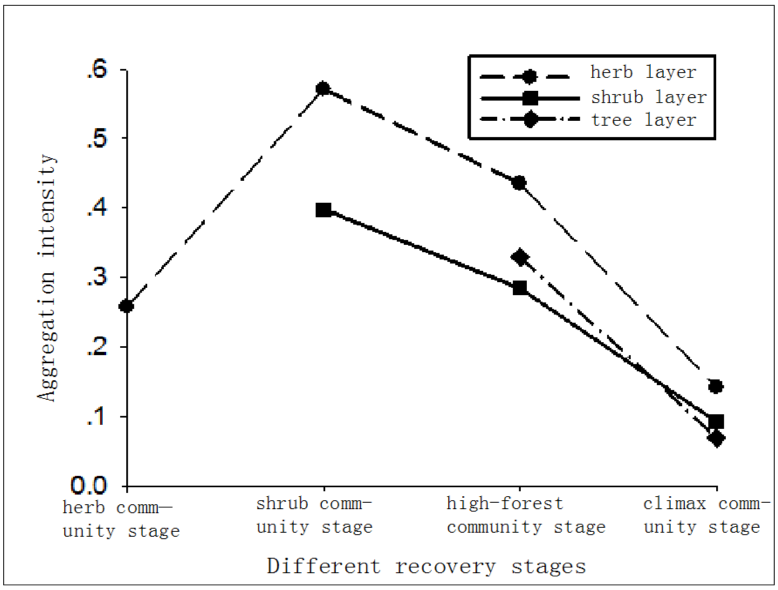

Figure 8. *Variation of aggregation intensity of dominant species*

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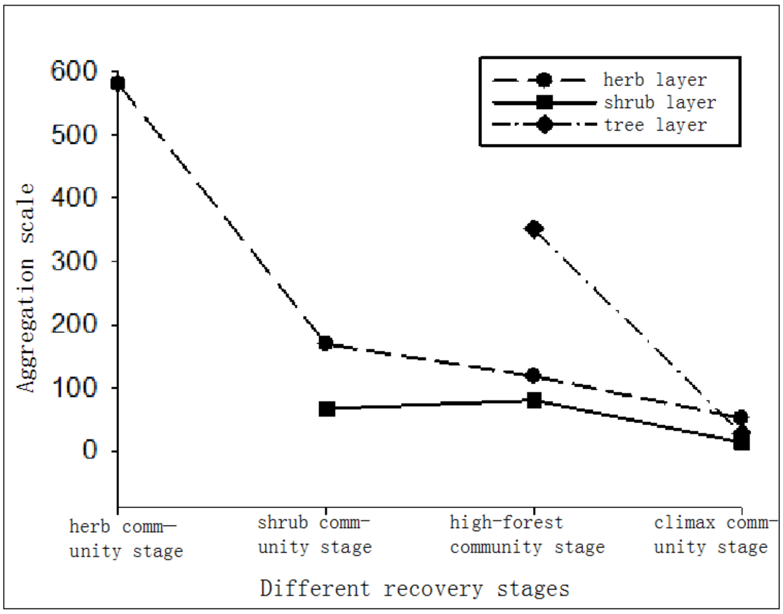

Figure 9. *Variation of aggregation scale of dominant species*

Therefore, the dominant species at constructive layer in early-mid succession stages are mainly in aggregated distribution, while in the late climax succession stage, they are mainly in random distribution and less are in aggregated distribution. This reveals that the dominant species of plant community at karst stony desertification areas in early stage has small aggregation scale, but larger aggregation intensity which gradually decreases with the succession. The largest aggregation scale of dominant species is relative smaller, therefore the allocation scale of plant community at karst stony desertification areas should be set small.

Disscussions

The natural succession of plant community follows certain law. The species composition and structure of plant community vary among different stony desertification areas. It has long been suggested that afforestation should be carried out according to local field condition. Through site classification and plant community investigation, it is believed that stony desertification control can be realized by closing the land for reforestation, but also by establishing artificial plant community with consideration on environment condition (site condition), plant ecological adaptability, community composition, community horizontal structure, and community vertical structure.

Liu Xin-ding studied the community composition in red-earth region of southern china during different recovery stages, finding that "the vegetation type and quantity both increase with the succession of recovery stages"(Liu, 2014), which is different from the situation at karst stony desertification area where the species vary in tendency of "increasing-decreasing-increasing". The major different is that the species quantity shows decreasing tendency in tree-shrub recovery stage, which is may be due to ecological environment difference as Guizhou belongs to krast stony desertification area with subtropical humid monsoon climate.

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Similar to karst stony desertification areas, the Loess Plateau of China include therophyte (T), cryptophyte (Cr),hemicryptophyte (H), chamaephyte (Ch), nanophanerophyte Ph(N), microphanerophytes Ph(Mi), mesophanerophyte Ph(Me), megaphanerophyte Ph(Ma), Vine plants (EP), evergreen plant (E)，deciduous plant (D). However, the dominant speice at Loess Plateau of China in the early recovery stage is perennial hemicryptophyte, which is different from therophyte at karst stony desertification areas. This indicates that the vegetation at Loess Plateau of China is influenced by typical temperate climate (Zhang, 2007). Due to the difference in climate and habitat characteristics, the adaptive ways of plant to environment between two area are significantly different, therefore, different vegetation restoration ways should be implemented.

Conclusions

At karst stony desertification area during early recovery stage when the environment is harsh, species that can be adapted to the environment are mainly light, drought-tolerant pioneer species, such as annual herb plants; with the successions of stages, woody plants gradually increase in a variation tendency of "increasing-decreasing-increasing". Till to shrub forest stage, the quantity of woody plants reach the maximum value, and the variation of species composition of whole community is identical to variation of species composition of woody plants.

Five different stony desertification areas all include therophyte (T), cryptophyte (Cr), hemicryptophyte (H), chamaephyte (Ch), nanophanerophyte Ph(N), microphanerophytes Ph(Mi), mesophanerophyte Ph(Me), megaphanerophyte Ph(Ma), Vine plants (EP), evergreen plant (E)， deciduous plant (D). However, there is difference in life form composition ratio during different succession stages. In the early succession stage, *herbaceous* plant is the dominant species; with the succession of stage, the *herbaceous* plant gradually decreases, and then increases slowly in climax stage.

During natural succession stages, the cover degree, predominance degree, and density of plant community represents the horizontal structural feature of karst stony desertification area. The cover degrees of plant community at all stony desertification areas are insignificantly different, which is around 80%. Moreover, the cover degrees of herb, shrub, tree layer all reach the maximum when they are in constructive layer. The predominance degrees of community at rough valley, fault basin, and plateau of karst stony desertification area are in variation of "increasing-decreasing" while that at canyon and peak-cluster depression of karst stony desertification are in variation of "increasing-decreasing-increasing". The density of shrub layer is in variation of "increasing-decreasing-increasing", while that of tree layer in increasing variation.

The dominant species at constructive layer in early-mid succession stages are mainly in aggregated distribution, while in the late climax succession stage, they are mainly in random distribution and less are in aggregated distribution. This reveals that the dominant species of plant community at karst stony desertification areas in early stage has small aggregation scale, but larger aggregation intensity which gradually decreases with the succession. The largest aggregation scale of dominant species is relative smaller, therefore the allocation scale of plant community at karst stony desertification areas should be set small.

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ESTIMATES OF CROSSBREEDING PARAMETERS FOR GROWTH AND CONFORMATION TRAITS IN NIGERIAN INDIGENOUS AND EXOTIC PIG BREEDS

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Abstract. Information on the crossbreeding parameter estimates involving Nigerian Indigenous pigs and exotic breeds are rare, as well as the best combination for improved growth and conformation traits. A 3×3 diallel cross was conducted among three breeds of pigs – Large White (WW), Landrace (RR) and Nigerian Indigenous pig (NN) breeds. Growth traits measured were body weight at birth (BWB), weaning (BWW at 6 weeks), maturity of 20 weeks (BW20) and average daily gain (ADG up to 20 weeks), while conformation traits were heart girth (HG), height-at-withers (HW), and body length (BL) all at 20 weeks of age. Significant heterotic and maternal effects were reported for BWW, BW20 and ADG. Also significant specific heterosis was recorded only for BW20, with no significant reciprocal effect for the other growth traits measured. Significant heterotic effects for HG and BL were also recorded, while maternal effect was significant only for BL. Significant specific heterosis was recorded for HG, while reciprocal effect was significant only for HW and B. This implies that for growth traits, RR, WW and NN breeds had the best combining ability for BWW, BW20 and ADG respectively with a high positive average heterosis of 0.06, 2.53 and 1.58. This shows that NN breed could elicit attractive economic and environmental benefits when crossbred with exotic ones, in terms of growth traits, as shown by the significant crossbreeding parameter values reported.
Keywords: *diallel cross, heterosis, maternal effect, reciprocal effects, pig production*

Introduction

Various attempts had been made to improve pig strains by identifying the best combining ability in terms of heterosis, maternal and reciprocal effects using diallel cross (Fernandez et al., 2002; Garcia-Casco et al., 2012; Ibanez-Escriche et al., 2014). The main purpose of crossing is to produce superior crosses to improve fitness, conformation and fertility traits, and to combine different characteristics in which the crossbreds were valuable (Balaguer, 2014). Improvement of different traits in pigs have been attempted using cross breeding estimates; such as carcass characteristics (Sutha et al., 2015), semen qualities (Gonzalez-Pena et al., 2015), reproduction and growth traits (Hsu and Johnson, 2014), fat deposition and fatty acid profiles (Ibanez-Escriche et al., 2016). The estimation of crossbreeding effects (direct genetic effect, heterotic effect, maternal effect and reciprocal effect, combining ability - General (GCA) and Specific (SCA)) therefore are of major importance. The testing of populations to attain evaluation of their combining ability requires systematic crossbreeding design. The diallel crossing is one such design. A diallel cross is a set of possible combinations between lines, breeds or general populations (Jakubec et al., 1987). Desirable characteristics of different breeds can be utilized if some breeds can be identified as good maternal breeds and others as good paternal breeds.

There is dearth of information on the crossbreeding effects of Nigerian indigenous pigs with exotic ones, particularly on estimates of crossbreeding parameters in such mating scheme. Duro et al. (2015) attempted the use of diallel cross method involving Nigerian indigenous pig and two exotic breeds to evaluate the effects of cross, parity and sex on bodyweight and morphometric traits among them and these estimates lacked information on crossbreeding parameters. This study is, therefore, aimed at estimating the crossbreeding parameters on growth and conformation traits in a complete diallel cross involving Nigerian indigenous, Large white and Landrace breeds, which are used mainly for production in Nigeria.

Materials and Methods

Study location

This research was carried out at the piggery unit of the Teaching and Research Farm of the Federal University of Technology Owerri, Imo State Nigeria between 2009 and 2014. The facility lies between Latitudes 4^0 04' and 6^0 03' N and Longitudes 6^0 15' and 8^0 15' W. The mean annual rainfall is 2500mm, temperature ranges from 26.5 to 27.5^0 C, and humidity ranges from 70 to 80%.

Animals and experimental design

A complete 3 × 3 diallel cross involving three breeds of pigs, namely Large White (WW), Landrace (RR) and Nigerian Indigenous pig (NN) breeds was conducted. The resultant possible mating combinations included 3 purebreds (WW, RR and NN) and 6 crossbreds (WR, WN, RW, RN, NW, and NR). The base population includes 5 sires per breed and 10 dams per breed which resulted in a total of 148 offsprings farrowed (*Table 1*).

Table 1. *Mean (±SEM) for the some growth traits measured*

Crosses[2]	Number from birth- weaning	Mean traits[1]±SEM		
		BWB (kg)	BWW(kg)	ADG (g)
W×W	25	1.42±0.06	7.25±0.36	138.81±28.74
R×R	24	1.53±0.07	7.37±0.30	140.05±27.62
N×N	21	0.92±0.03	5.36±0.15	105.71±18.07
W×R	17	1.22±0.04	7.51±0.34	149.76±16.76
W×N	13	0.82±0.07	5.94±0.46	121.90±19.72
R×W	15	1.10±0.04	6.25±0.33	122.61±27.51
R×N	12	1.02±0.04	7.12±0.29	145.24±23.05
N×W	10	1.01±0.05	6.85±0.39	139.05±18.43
N×R	11	0.94±0.03	6.66±0.34	136.19±22.91
Parity[3]				
1	65	1.18±0.04	6.62±0.20	129.52±18.75
2	83	1.15±0.03	6.82±0.16	135.02±22.15
Sex[4]				
1	73	1.13±0.04	6.60±0.18	130.24±19.75
2	75	1.20±0.04	6.87±0.17	135.50±27.09
TOTAL	148			

[1]BWB = Bodyweight at birth, BWW = Bodyweight at 6 weeks weaning, ADG = Average daily gain upto 20 weeks, SEM = Standard error of mean.
[2]Breed of boar x Breed of sow, W= Large White, R= Landrace, N = Nigerian Indigenous.
[3]Parity 1 = first parity, Parity 2 = second parity.
[4]Sex 1 = Female, Sex 2 = Male.

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The method of identification was through the use of ear notching identical for each pure bred and cross bred genetic groups. The initial crossbred population was generated between 2009 and 2011 (Okoro, 2012), while the pure population was generated between 2011 and 2014. The data used were records from 2 parities per cross in the experiment from the population bred at the Teaching and Research farm. The number of records, cross distribution and the summary statistics for all the traits measured are shown in *Tables 1* and *2*.

Table 2. *Mean (±SEM) for a growth and conformation traits measured*

Crosses[2]	Number at maturity	Mean traits[1]±SEM			
		BW20 (kg)	HG(cm)	HW(cm)	BL(cm)
W×W	24	57.22±1.54	52.88±2.08	48.65±1.65	65.71±1.85
R×R	23	58.26±1.80	50.13±1.47	48.35±1.63	70.26±1.47
N×N	20	42.48±0.97	46.55±1.09	41.80±1.08	60.80±0.94
W×R	15	50.67±1.35	50.00±1.53	47.40±0.99	62.60±1.03
W×N	12	44.52±1.32	46.75±1.38	45.70±1.15	57.17±1.75
R×W	14	52.53±1.14	53.22±0.92	48.74±0.93	72.29±0.91
R×N	11	52.76±0.98	52.86±1.05	51.03±0.86	71.86±1.48
N×W	9	46.68±1.14	48.33±2.07	47.61±1.99	64.44±2.63
N×R	11	46.15±0.95	48.36±1.52	45.68±1.55	70.36±1.42
Parity[3]					
1	62	51.45±1.12	49.18±0.94	47.09±0.85	65.87±1.09
2	77	50.79±0.86	50.76±0.71	47.14±0.67	66.32±0.81
Sex[4]					
1	67	50.19±1.00	49.97±0.83	46.48±0.82	65.85±0.92
2	72	51.91±0.94	50.13±0.80	47.72±0.67	66.37±0.94
TOTAL	139				

[1]BW20 = Bodyweight at 20 weeks maturity, HG = Heart girth at 20 weeks, HW = Height at withers at 20 weeks, BL = Body length at 20 weeks, SEM = Standard error of mean.
[2]Breed of boar x Breed of sow, W= Large White, R= Landrace, N = Nigerian Indigenous.
[3]Parity 1 = first parity, Parity 2 = second parity.
[4]Sex 1 = Female, Sex 2 = Male.

Management of experimental animals

The parent animals were fed *ad libitum* on concentrates containing 17% crude protein (CP) and 2,480 Kcal/kg energy. A concentrate feed containing 24% CP and 2,500 Kcal/kg energy was also used to flush the sows during the gestation period to enhance nutrients for embryonic and foetal development. Other management practices of animals in the study are in accordance with descriptions in study by (Duro et al., 2015).

Parameters measured

The parameters measured were growth traits and conformation traits taken from the purebreds and the crossbred progenies at the various stages of growth which includes:
1) Growth traits
   i) Birth weight taken at birth of the piglets using 50 kg weighing scale ® Salter England);
   ii) Weaning weight taken at 6 weeks after birth using 200kg bridge weighing scale ® Global Universal, England);

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iii) Maturity weight taken at 20 weeks post birth using 200kg bridge weighing scale ® Global Universal, England);
iv) Average Daily gain taken daily as the difference between initial weight and current weight taken from birth to maturity at 20 weeks.

2) The conformation traits taken at 20 weeks post birth which includes:
i) Heart Girth (HG): Measured as the circumference of the animal body taken immediately posterior to the shoulder, using a tailor's tape;
ii) Height at Withers (HW): Measured as the distance from the highest point on the dorsum of the animal to the ground surface, at the level of the front feet, using a tailor's tape;
iii) Body length (BL): Measured as the distance from the point of the scapular to the pin bone of the tail base, using a tailor's tape.

Statistical analysis

The following statistical model according to (Jakubec et al., 1987) was used to analyse the diallel cross:

$$y_{hijk} = \mu^B + a_h^B + p_{lii}^B + g_{2i}^B + g_{2j}^B + c_{2ij}^B + m_{2j}^B + r_{2ij}^B + e_{ijk}^B \qquad \text{(Eq.1)}$$

Where

y_{hijk} = the k-th observation on the progeny of a mating of a dam from the j-th line with a sire of the i-th line in the h-th type of breeding (purebred or crossbred, h = 1,2), μ^B= mean of the purebred and crossbreds, a_h^B = effects common to all progeny of the h-th type of breeding (purebred or crossbreds); similar to average heterosis ($\bar{h}^A$) in model A (Eisen et al., 1983), p_{lii}^B = effect common to all progeny of a mating between a dam of the i-th line and a sire of the i-th line; g_{2i}^B= general combining ability of the i-th line, c_{2ij}^B= specific combining ability of the ij-th line cross, m_{2j}^B = maternal genetic effect of the j-th line, r_{2ij}^B = residual reciprocal effect in the cross ij, e_{ijk}^B = random error. The subscript 1 denotes that the effects are measured only among the purebred progeny, while the subscript 2 denotes that the effects are measured only among the crossbred progeny.

Restrictions imposed on the parameter estimates are:

$$\sum_h a_h^B = \sum_i p_{lii}^B = \sum_i g_{2i}^B = \sum_j m_{2j}^B = \sum_i c_{2ij}^B = \sum_j c_{2ji}^B = \sum_i r_{2ij}^B = \sum_j r_{2ji}^B = r_{2ij}^B + r_{2ji}^B = 0 \qquad \text{(Eq. 2)}$$

The least squares estimators of the parameters are as shown in (Jakubec et al., 1987), where model A is the model developed by (Eisen et al., 1983) while model B is developed by (Harvey, 1960) and extended by (Jakubec et al., 1987). The choice of this model (Model B) is because it is the most appropriate when the parental lines are less than 4. With all the crossbreeding parameters being the major interest in this study, relating some aspects of model A to model B according to (Jakubec et al., 1987) was necessary in order to capture all the parameters of heterosis (direct, line and specific heterosis) and maternal effects (direct and maternal genetic effects). The equations that relate parameters in model A to model B are as shown below:

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$$\bar{\mu}^B = \bar{\mu}^A + {}^1/_2\,\bar{h}^A \quad \text{(Eq.3)}$$

$$\bar{a}_1^B = -\,{}^1/_2\,\bar{h}^A \quad \text{(Eq.4)}$$

$$\bar{a}_2^B = {}^1/_2\,\bar{h}^A \quad \text{(Eq.5)}$$

$$\bar{p}_{1ii}^B = \bar{v}_i^A + \bar{m}_i^A \quad \text{(Eq.6)}$$

$$\bar{g}_{2i}^B = {}^1/_2\,\bar{v}_i^A + \bar{h}_i^A \quad \text{(Eq.7)}$$

$$\bar{c}_{2ij}^B = \bar{s}_{ij}^A \quad \text{(Eq.8)}$$

$$\bar{m}_{2j}^B = \bar{m}_j^A \quad \text{(Eq.9)}$$

$$\bar{r}_{ij}^B = \bar{r}_{ij}^A \quad \text{(Eq.10)}$$

The simple difference in the two models is in the estimation of the direct genetic effect and line heterosis in model A which are substituted with the term general combining ability in model B. The model A parameters for heterosis $\bar{h}^A$ and $\bar{h}_i^A$ were derived from model B parameters according to (Jakubec et al., 1987) thus;

$$\bar{h}^A = \bar{a}_2^B - \bar{a}_1^B \text{ or } \bar{h}^A = 2\,\bar{a}_2^B \text{ and } \bar{h}_i^A = \bar{g}_{2i}^B - \frac{\bar{p}_{1ii}^B - \bar{m}_{2i}^B}{2} \quad \text{(Eq.11)}$$

Therefore, heterosis and maternal effects were estimated from these expressions based on the number of parental lines, which is three, leading to all the parameters for heterosis captured. Where there were significant least squares estimate, the least significant difference (LSD) method was used to separate the means (SAS Institute, 2010).

Results

There were significant effects due to direct and maternal genetic effects, GCA, line heterosis and maternal effects on growth traits as estimated from equations 7, 8 and 9, at a probability levels of 95% and 99% (P<0.05 and P<0.01) (*Table 3*). However, significant specific heterosis was reported only for BW20 (P<0.01), and no significant reciprocal effect was reported for all the growth traits measured. The significant GCA translates to significant line heterosis on three growth traits measured viz BWW, BW20 and ADG up to 20 weeks (*Table 3*).

However, the values of crossbreeding parameters for the Large White genotype consistently expressed positive values for the growth traits, particularly at weaning, maturity and ADG, except for heterosis and maternal effects which expressed negative values (*Figs. 1B, 1C, 1D, 1E & 1F*).

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Table 3. *Least squares means and standard errors of crossbreeding parameter (Overall mean -μ, mean heterosis - h, direct & maternal genetic effects -p, general combining ability - g, line heterosis - h, maternal effects - m, specific heterosis - c and reciprocal effects - r)for growth traits*

	BWB (kg) 0days		BWW(kg) 42days		BW20 (kg)		ADG (g)	
Parameters	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
μ	1.16	0.49	6.69	2.24	49.19	16.03	132.78	20.78
h	-0.27	0.11	0.06	0.02	2.53	1.03	1.58	0.03
$a_1^{\#}$	0.14	0.06	-0.03	0.01	-1.27	0.02	-0.79	0.02
$a_2^{\#1}$	-0.14	0.05	0.03	0.01	1.27	0.015	0.79	0.015
p_{WW}	0.13	0.02	0.59[a]	0.26	2.43[a]	0.24	3.94[b]	0.57
p_{RR}	0.24	0.07	0.71[a]	0.29	3.00[a]	0.23	-0.77[c]	0.56
p_{NN}	-0.37	0.11	-1.01[b]	0.39	-8.42[b]	0.83	8.57[a]	3.56
Sig.	**ns**		*		**		*	
g_W	0.01	0.02	0.14[b]	0.24	1.69[a]	0.56	1.13[b]	0.79
g_R	0.16	0.05	0.44[a]	0.16	-3.53[b]	3.08	-4.23[c]	4.20
g_N	-0.20	0.06	0.17[b]	0.47	-5.78[b]	1.94	2.54[a]	0.87
Sig.	**ns**		*		*		*	
h_W	-0.04	0.02	-0.22[b]	0.13	0.58[a]	0.24	0.05[b]	0.02
h_R	0.03	0.017	0.215[a]	0.09	-5.88[b]	2.40	0.18[a]	0.05
h_N	0.03	0.02	0.74[a]	0.30	-2.32[b]	0.94	0.11[b]	0.08
Sig.	**ns**		*		**		*	
m_W	0.03	0.01	-0.12[b]	0.05	0.22[a]	0.09	0.92[b]	0.08
m_R	0.01	0.004	0.27[a]	0.16	-1.71[b]	0.70	0.77[b]	0.12
m_N	-0.04	0.02	-0.15[b]	0.06	-1.49[b]	0.61	1.75[a]	1.54
Sig.	**ns**		*		*		*	
c_{WR}	-0.04	0.013	0.50	0.17	8.41[a]	2.80	0.82	0.15
c_{WN}	0.08	0.026	-0.49	0.16	-1.62-	0.54	-1.85	1.47
c_{RN}	0.02	0.007	0.49	0.16	8.41[a]	2.79	1.86	1.06
Sig.	**ns**		**ns**		**		**ns**	
r_{WR}	0.02	0.006	0.33	0.10	-0.24	0.07	0.53	0.08
r_{WN}	-0.26	0.075	0.09	0.03	-4.5	1.30	0.79	0.18
r_{RN}	0.04	0.02	0.22	0.06	-0.22	0.06	1.35	1.33
Sig.	**ns**		**ns**		**ns**		**ns**	

[#]and[#1]= purebred and crossbred heterosis respectively. [abc] means on the same column within the same parameter but with different superscripts are significantly different. *=P>0.05, **=P>0.01.

For the conformation traits, there were significant direct and maternal genetic effects on HG and HW ($P < 0.05$) but none on BL ($P > 0.05$). GCA was significant on HG ($P < 0.01$) and BL ($P < 0.05$), resulting in a significant line heterosis on the two traits respectively but none significant on HW (*Table 4*). These are based on equations 7 to 11. Meanwhile, maternal effects and specific heterosis were significant on BL ($P < 0.05$) and HG ($P < 0.01$) respectively, while reciprocal effects were significant on HW ($P < 0.05$) and BL ($P < 0.01$) traits, respectively. The conformation traits also showed an inconsistent distribution of values for the crossbreeding parameters measured. Apart from heterosis, GCA and reciprocal effects in the Large White genotype reflected negative values throughout the period of the experiment, while the rest were all positive estimates of crossbreeding parameters (*Figs. 2A, 2B, 2C, 2D, 2E & 2F*).

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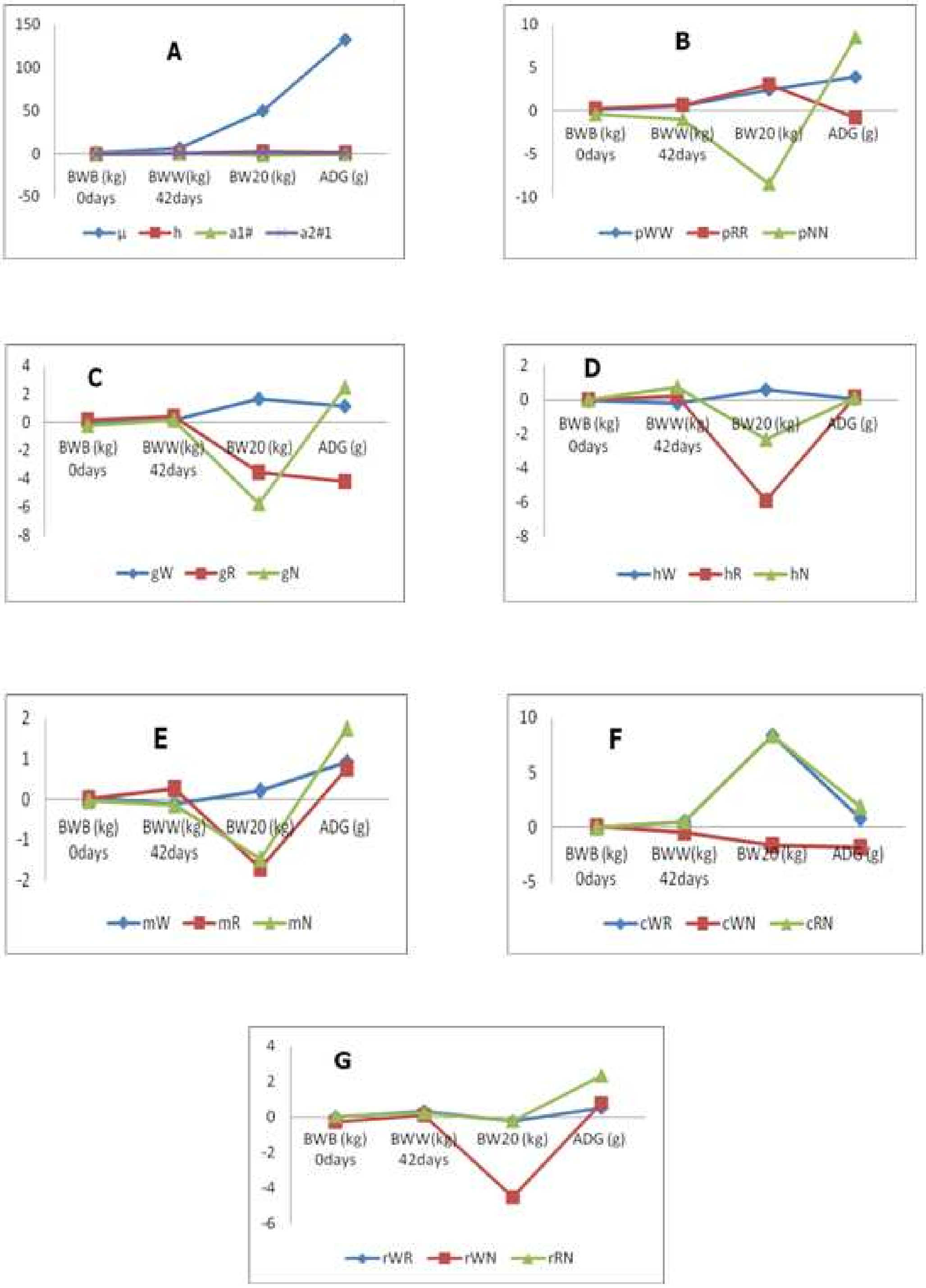

Figure 1. *Interactive graph showing the estimates of crossbreeding parameters for growth traits (body weight at birth – BWB, bodyweight at weaning – BWW, body weight at maturity of 20 weeks – BW20, & average daily gain up to 20 weeks – ADG); µ = Overall mean, h (A) = mean heterosis, p = direct & maternal genetic effects, g = general combining ability, h (D) = line heterosis, m = maternal effects, c = specific heterosis, and r = reciprocal effects*

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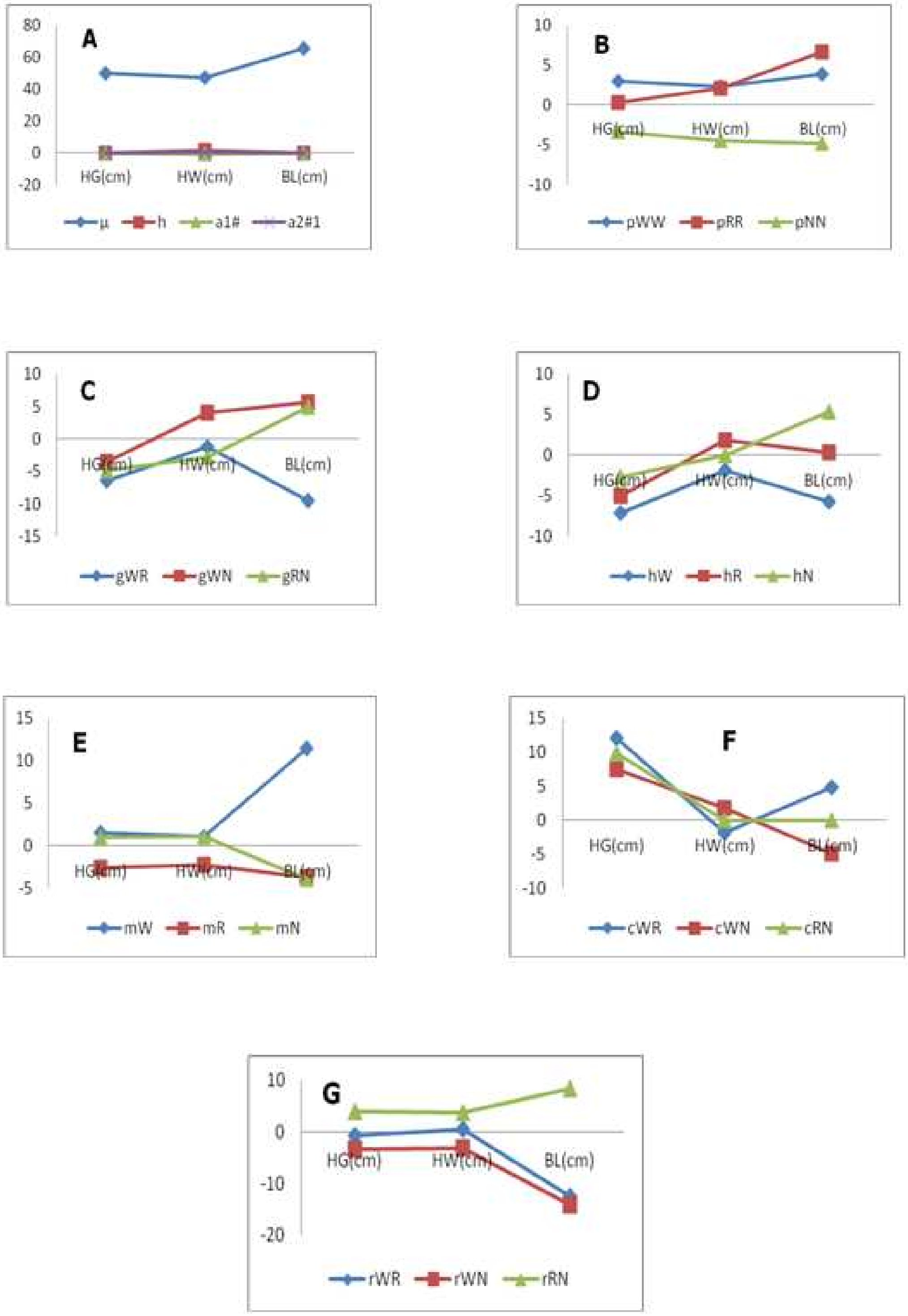

Figure 2. *Interactive graph showing the estimates of crossbreeding parameters for conformation traits (heart girt - HG, height at withers - HW, & body length – BL); μ = Overall mean, h (A) = mean heterosis, p = direct & maternal genetic effects,g = general combining ability, h (D) = line heterosis, m = maternal effects, c = specific heterosis, and r = reciprocal effects*

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Table 4. *Least squares means and standard errors of crossbreeding parameter (Overall mean -μ, mean heterosis - h, direct and maternal genetic effects - p, general combining ability - g, line heterosis - h, maternal effects - m, specific heterosis - c and reciprocal effects - r) for conformation traits*

	HG(cm)		HW(cm)		BL(cm)	
Parameters	Mean	SEM	Mean	SEM	Mean	SEM
μ	49.89	16.64	46.99	15.71	65.53	21.89
h	0.07	0.03	1.43	0.58	-0.13	0.05
a1[#]	-0.04	0.03	-0.715	0.29	0.07	0.025
a2[#1]	0.035	0.03	0.72	0.30	-0.07	0.03
p_{WW}	3.03[a]	1.06	2.38[a]	0.82	3.90	6.84
p_{RR}	0.28[b]	1.87	2.08[a]	2.15	6.67	4.52
p_{NN}	-3.30[c]	1.63	-4.47[b]	2.09	-4.79	1.85
Sig.	*		*		**ns**	
g_{WR}	-6.42[c]	3.12	-1.20	0.39	-9.50[b]	3.43
g_{WN}	-3.55[a]	2.44	4.02	1.24	5.59[a]	1.66
g_{RN}	-4.82[b]	1.71	-2.82	0.63	4.87[a]	2.29
Sig.	**		**ns**		*	
h_W	-7.13[c]	2.91	-1.85	0.62	-5.73[c]	2.34
h_R	-4.97[b]	2.03	1.86	0.62	0.39[b]	0.16
h_N	-2.68[a]	1.09	-0.01	0.006	5.30[a]	2.16
Sig.	*		**ns**		*	
m_W	1.60	0.65	1.08	0.44	11.43[a]	4.67
m_R	-2.57	1.05	-2.23	0.91	-3.73[b]	1.52
m_N	0.97	0.40	1.15	0.47	-3.92[b]	1.60
Sig.	**ns**		**ns**		*	
c_{WR}	12.14[a]	4.05	-1.85	0.62	4.93	1.64
c_{WN}	7.56[b]	2.52	1.87	0.62	-4.89	1.63
c_{RN}	9.85[a]	3.28	0.01	0.006	0.02	0.007
Sig.	**		**ns**		**ns**	
r_{WR}	-0.64	0.18	0.48[b]	0.14	-12.55[b]	3.62
r_{WN}	-3.35	0.97	-3.19[c]	0.92	-14.15[b]	4.08
r_{RN}	3.85	1.11	3.76[a]	1.09	8.40[a]	2.42
Sig.	**ns**		*		**	

[#and#1]= purebred and crossbred heterosis respectively, [abc] means on the same column within the same parameter but with different superscripts are significantly different. *=P>0.05, **=P>0.01.

Discussion

The observed average heterosis of -0.27kg, 0.06kg, 2.53kg and 1.58g/day for BWB, BWW, BW20 and ADG, respectively, for the growth traits shows there was effective heterosis in the diallel cross, although BWB exhibited negative heterosis. However, the positive average heterosis exhibited by other traits might explain the mostly significant crossbreeding parameters expressed subsequently in the traits. The significant direct and maternal genetic effects on growth traits imply that these traits are influenced significantly by genotypes. The growth traits BWW, BW20 and ADG were influenced more by genotype in breeds RR, RR and NN respectively due to the high mean values they exhibited. Estimates of genetic effects are useful to evaluate pig breeds and to

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develop efficient crossbreeding systems (Hidalgo et al., 2015). Therefore, when selecting purebred lines for crossbred performance, the potential use of dominance, imprinting and breed-specific effects could be considered an alternative to improve the efficiency of crossbreeding and selection.

Hildago et al. (2015) reported non-significant direct genetic effect on weight at all ages for pure bred cross, whereas maternal genetic effects differed significantly for weight at 14, 28 and 154 days of age for purebreds using genomic breeding value accuracy prediction methods. Meanwhile, the crossbred had a significant effect of direct genetic effect on weight at 56, 70 and 154 days while for the maternal genetic effect; it differed significantly at birth, 28 and 56 days. Hsu and Johnson (2014) reported highly significant direct maternal effects on 180-d weight (WT180) and longissimus muscle area (LMA) and decreased back fat (BF10) in a Large White – Landrace composite population which favored the Large white breed.

For the significant GCA and its composite; line heterosis, it implies that for the growth traits, BWW, BW20 and ADG, the breeds, NN, WW and RR had a positive line heterosis of 0.74, 0.58 and 0.18 for each breed and GCA values of 0.44, 1.69 and 2.54 for RR, WW and NN breeds respectively. Kurnianto et al. (2010) in a diallel study involving Duroc, Yorkshire and Landrace breeds of pigs estimated GCA for growth traits such as birth weight, average daily gain (ADG), post weaning ADG and body weight at 42 days and found it to be significant and in favour of Duroc breed, due to the highest value reported which implies a significant line heterosis. In a similar vein, Garcia-Casco et al. (2012) in a complete diallel cross scheme with 4 ancient lines of Iberian pigs reported a significant heterotic effect on weight at 420 days of age for the different crosses, while a non-significant heterotic effect for daily growth rate inferred in the first 5 combinations, but the remaining combinations had significant heterosis (+66g/d). Sutha et al. (2015) also reported that in a diallel cross involving Duroc, Yorkshire and Landrace gilts, offspring from Duroc boars were most efficient in feed utilization, and a synthetic three way pig cross was achieved with very high lean weight. They also.reported high heterotic gains in carcasses with less loin fat, a thicker loin muscle, a higher estimated lean yield and a better classification index. These results are in agreement with the findings in this study and by Ibanez-Escriche et al. (2016) in which heterosis effects were relevant for the Retinto and Entrepelado pig breed crosses (approximately 16% of the trait), which could be valuable for a crossbreeding system involving these lines.

The significant maternal effects with high positive values of 0.27, 0.22 and 1.75 for RR, WW and NN breeds respectively, implies that the traits BWW, BW20 and ADG are influenced more by the dams respectively. Gonzalez-Pena et al. (2015) in a three and four way crossbreeding for swine production reported a significant maternal effect for average daily gain (ADG) backfat (BF), feed efficiency (FE), and carcass lean % (LEAN) due to semen traits. Silva et al. (1996) estimated the maternal effects on bodyweight at birth, 21, 35, and 77 days in a full diallel crosses involving Duroc, Landrace, Yorkshire and Large White pigs and reported a significant maternal effect at birth, 21 days and 35 days of age. Also, Hsu and Johnson (2014) reported that crossbreeding and selection for growth traits along with litter size selection at 19 to 28 days resulted in responses consistent with the selection applied and the heritability of the trait on maternal effects. Also pig body weights appeared to be positive from weaning to 154days in Yorkshire breed dams, while Duroc dams seemed to perform poorly throughout. The positive influence of Yorkshire gilts on pig size was somewhat

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unexpected in view of the fact that Yorkshire gilts also had the largest crossbred litters. Thus, Yorkshire gilts not only raised slightly larger litters but also weaned heavier pigs and their advantage carried through to 154-day weight.

Also, the significant specific heterosis for BW20 implies that lines for the best specific heterosis are combinations of W×R and R×N with values of 8.41kg respectively and the worst combination being W×N with -1.62kg. Kurnianto et al. (2010) also reported a significant SCA in favour of Yorkshire × Landrace breed cross for all the growth traits studied in a diallel cross involving Duroc, Yorkshire and Landrace breeds of pigs.

For the conformation traits, significant direct and maternal effects for HG and HW imply that these traits were influenced by the genotype than phenotype. The WW breed exhibited the highest mean value for both traits (3.03kg and 2.38kg) respectively. With the RR breed being phenotypically characterized by longer body frame than WW and NN breeds, it was expected that it would have expressed a significantly higher BL. This may be explained by the inherent expression of the trait which might not be only heterotic, but also epistatic. But for the GCA and its composite - line heterosis; there is significant heterotic effect in HG and BL, with BL having the highest mean value (5.59cm) in W×N cross. In the line heterosis however, BL trait was exhibited more in RR breed, with the highest mean value of 6.67cm, showing that the trait might be inherent in the breed naturally and expressed epistatically instead of in heterosis. Meanwhile, HG trait exhibited negative mean values for both GCA and line heterosis, even though significantly influenced. This might imply that the traits were negatively influenced in the three breeds analyzed. The maternal effects are also significant only in BL trait, while HG and HW were not. This is more exhibited in the WW breed, as shown in its mean value of 11.30cm, which implies that that the dams of this breed enhances the trait in swine. The specific heterosis also showed that only HG trait was significantly influenced while HW and BL were not with the W×R combination exhibiting the highest value (12.14cm) while W×N was the least. This implies that HG trait is more expressed in the W×R combination. Whereas for the reciprocal effect, HW and BL traits were significantly influenced, while HG was not. The combination of R×N was consistently highest in mean values for both HW and BL traits, showing that the reciprocal cross will be influenced in the same way as the main cross.

Conclusion

Effective combination of local breeds of animals such as Nigerian indigenous breeds in crossbreeding programs to improve growth and reproductive traits has been advocated, as shown by the advantage of Chinese breeds used in production due to its high prolificacy (Rothschild, 1996). The Nigerian indigenous pigs in this study have shown its ability to enhance the specific heterosis with the Landrace breed in improving bodyweight at 20 weeks, which could have major underlying advantage in commercial swine production. This study which is aimed to understand the genetic underpinnings of the breeds as well as the best combination to enhance growth and conformation traits, may also enhance the adaptability of the crossbreds in the harsh tropical environment where the exotic breeds are not used to.

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SIMULATION OF SOYBEAN CANOPY NUTRIENT CONTENTS BY HYPERSPECTRAL REMOTE SENSING

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Abstract. Precision fertilizer management could help reduce farming costs and maintain production sustainability in current cropping systems. Soybean is a major oil crop and to improve temporal and spatial fertilizer application to demand variations, soybean canopy nutrient status was diagnosed by the hyperspectral remote sensing techonology. First, field canopy spectral reflectance was characterized during key developmental stages with three levels of fertilizer treatments in northeastern China. Then, foliar nitrogen (N), phosphorus (P) and potassium (K) contents were quantified and analyzed for correlation with transformed spectral data formats including reciprocal, logarithm and derivatives, red edge parameters and vegetation index. Last, simulation models for soybean canopy nutrient status (total N, P and K) were constructed. The simulation model (y= -19.153x+3.1114) using second derivatives of spectral data at 432 nm was proved to significantly correlate the predicted value with measured total N content (r=-0.7829, p<0.01; RE=0.1713). The first derivative-derived models y=-0.2939x+0.5889(r=-0.6172, p<0.01; RE=0.2428) at 909 nm and y=-0.4157x+1.874(r=-0.5631, p<0.01; RE=0.1345) at 908 nm produced most accurate prediction for total P and K respectively. Models reported in this work were top selections for the simplicity and practicality in predicting soybean nutrient and growth status.
Keywords: *soybean, nutritional status, predictive modeling, remote sensing, canopy reflectance*

Introduction

To meet increasing needs of food supply from the fast growing global population, high cropping yields have to be achieved and be even further increased. Since plant growth, development and productivity depend on the availability of nutrients, intensive use of fertilizers has become common agronomic practices of current farming systems especially in low productive regions. However, excessive application of fertilizers has caused major detrimental impacts on the ecosystem and increased costs for both producers and consumers. In this regard, there is a clear need of more reasonable and "intelligent" use of fertilizers to help maintain environmental and economic sustainability of the agricultural production (Chen et al., 2014), which is a main aspect of precision agriculture (PA) (Gebbers and Adamchuk, 2010). Precision fertilization allows a finer degree of fertilization responding to intra-field variability in crops such as different soil conditions and the "heterogeneous" plant growth status so that fertilization efficiency can be improved and productivity is "intensified" (Lindblom et al., 2017).

Plant growth status can be reflected by outward structural characteristics and internal chemical compositions. Hyperspectral remote sensing is a technology used for the collection of information of contiguous high-resolution electromagnetic radiation emitted from an object so that to recognize and locate the target and reveal its natural properties. In recent years, the hyperspectral agricultural remote sensing technology has been used to

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predict crop water content (Holben et al., 1983; Su et al., 2010; Liu et al., 2012), chlorophyll content (Madeira et al., 2000; Tang et al., 2011; Curran et al., 1999), leaf surface area index (Bouman et al., 1992; Danson and Plummer, 1995; Feng et al., 2009) and other biophysical parameters (Vergara-Díaz et al., 2016) and major nutrient elements of plants (Femandez et al., 1994; Cheng et al., 2011; Yi et al., 2014), monitoring micronutrient and other nutrient status of crops (Masoni et al., 1996; Wang et al., 2012).

A combination of techniques suitable for remotely sensing foliar Nitrogen (N) in semiarid shrublands – a capability that would significantly improve our limited understanding of vegetation functionality in dryland ecosystems (Mitchell et al., 2012). In A. Zerger study introduced that environmental sensor networks for vegetation, animal and soil sciences (Zerger et al., 2010). The ratio vegetation index (RVI) between the spectral reflectance from the near-infrared and visible band ranges can be used to construct a hyperspectral simulation model for soybean leaf area index (Zhang et al., 2005). Advance on application of of hyperapectral technology in detection of crop was proposed (Liu et al., 2013). Findings from these large numbers of research efforts have provided the theoretical bases for incorporating the remote sensing technology in precision fertilization by diagnosing plant nutrient status.

Crop species each may have its own characteristic spectrum; the information from visible light, near infrared light, and short infrared region can be used to produce biomass at high accuracy (Shibayama et al., 1989). Study by a Chinese group discovered that in a soybean crop, the above-ground fresh weight has a strong correlation with the spectral signatures in the range of 760~1050nm, and RVI can be used to accurately predict soybean above-ground biomass yield (Song et al., 2005; Chen et al., 2010). The canopy reflectance indices measured at early flower stage of cotton growth could serve as input to a crop growth model for predicting potential yield loss (Zhao et al., 2007).

Chlorophyll content and leaf spectral characteristics have a very strong correlation (Madeira et al., 2000). Studies found that soybean chlorophyll A, B contents have a negative relationship with canopy reflectance in the visible band range, and in the near-infrared wavelength, and it changed to positive in the red edge band region (Song et al., 2006). At 536, 577, 611, 680, 705 nm wavelengths, the first derivative of spectral reflectance has a significant correlation with chlorophyll content (Chen et al., 2012). In Tang study proposed to use the Neural Network Model to estimate stable soybean canopy chlorophyll content.

Spectral measurements are useful for estimating the nitrogen status of crops, thereby enabling site-specific fertilizing in precision farming systems (Mistele and Schmidhalter, 2008). Soybean is one of the most cultivated oil crop worldwide and especially in northeastern regions of China in terms of land area and production (Zhang et al., 2014). Presently, spectral remote sensing technology is mainly used in soybean field to analyze biomass yield, leaf surface area and chlorophyll content. Plant nutrient diagnosis studies, in particular of P and K, have not been investigated. In this study, the technology and theory in remote sensing and the ground remote sensing were applied to develop models for the evaluation of soybean plants, in combination with the hyperspectral non-invasive monitoring technology and field data synchronization collection method, and with the aid of statistical analysis methods. The objective was to explore the major agronomic parameters, and leaf nutrient contents, and its relationship with reflectance spectrum. The characteristic spectral signatures were extracted for each plant growth parameter, and to construct simulation models for plant growth using canopy reflectance spectral data. Findings from this study will provide theoretical bases for real time diagnosis and accurate management of soybean fields in Northeast China.

Materials and Methods

Materials and Experimental Design

The experiment was performed on the experimental station at Shenyang, China (41°48′11.75″N, 123°25′31.18″E) in 2013. The soil of experimental sites were similar typical cropping soil in northeastern China which contained a total content of 2.65 g kg^{-1} N, 0.083 g kg^{-1} P, 75 mg kg^{-1} alkali-hydrolysable nitrogen, 0.07 mg kg^{-1} available P, 149.56 mg kg^{-1} available K, 18 g kg^{-1} organic matter. Liaoning No.14, a semi-determinate and Liaoning No.15, a determinate cultivar were chosen as experimental varieties. Seeds were sown on the 18^{th} of Maywith a density of 165, 000 plants hm^{-2}. Three P levels (P_2O_5), at 0, 5.5, 11 kg hm^{-2}, were designated as P0, P1, P2 respectively. Each plot (L 7 m x W 4 m) was used for one soybean variety with one P treatment. Every variety and treatment combination was repeated for three times and the total eighteen plots were randomly arranged. The same field management procedures were adopted for all experiments.

Collection of canopy spectral data

A portable spectrometer (ASD, USA) was used to collect canopy spectral reflectance. The instrument was operated with a 512 *element photo-diode array* (PDA) probe within wavelength spectrum of 325 ~ 1075 nm. The scanning was conducted according to the operational instruction for ASD as: spectral resolution was 3 nm; scan time interval was 17 ms; spectral sampling interval was 1.5 nm; the probe was placed vertically over the canopy at a field angle of 10°; the distance from probe to the top of the canopy was 1 m. The instrument was calibrated against internal reference prior to each measurement. Measurements were taken during key soybean developmental stages including branching, early flowering, peak flowering, pod setting and seed bulging stages. To control variation from field conditions, measurements were only taken on sunny days from 10 AM to 14 PM (local time). Each spectral reflectance data was an average of twenty measurements on a single plot.

Analysis of N, P and K contents in soybean leaves

After taking spectral measurements, fully expended leaves from the canopy top were cut without petiole and frozen immediately in liquid nitrogen. On each plot, twelve leaves collected from six plants were oven-dried (105 °C for 0.5 hour followed by 80°C for 2 days) then ground into fine powder for mineral content analysis. Sample digestion was carried out using the H_2SO_4-H_2O_2 method. The total nitrogen content was quantified using the classic Kjeldahl method. The total P content was quantified using the Mo-Sb- Vc- colorimetric method and the atomic absorption spectroscopy was used for K quantification.

(Leaf area index was measured but not included in this manuscript.)

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Processing of spectral data

(1). The first and second derivative spectrum

The reflectance spectra were transformed into the first derivative spectral revalues using the following equation:

$$\rho'(\lambda_1) = [\rho(\lambda_{i+1}) - \rho(\lambda_{i-1})]/2\Delta\lambda \quad \text{(Eq.1)}$$

λ_i is the wavelength of each channel (band); $\rho'(\lambda_i)$ is the first derivative spectrum of each band;

$\Delta\lambda$ is the interval difference from λ_{i-1} to λ_i

The second derivative spectrum was calculated by using the first derivatives as input data into the above equation.

(2). Calculation of red edge parameters

The red edge parameters were calculated for bands corresponding to the first derivative spectrum maximum within the red edge range (680 ~ 760 nm). The red edge slope is the first derivative spectra collected from the maximum peak area in the red edge range (680 ~ 760 nm). The red edge peak area is the size of the area surrounded by the first derivative spectra in the 680 ~ 760 nm range.

(3). Calculation of common vegetation indices

Common vegetation indices were calculated according to equations listed in the *Table 1.*

Table 1. *Formula for calculating common vegetation indices.*

Index	Abbreviation	Equation	Reference
Ratio vegetation index	RV	$RVI = \frac{\rho_{NIR}}{\rho_{RED}}$	Pearson et al.（1972）
Difference vegetation index	DVI	$DVI = \rho_{NIR} - \rho_{RED}$	Jordan（1969）
Normalized difference vegetation index	NDVI	$NDVI = \frac{\rho_{NIR} - \rho_{RED}}{\rho_{NIR} + \rho_{RED}} = \frac{RVI - 1}{RVI + 1}$	Rouse et al.（1974）
Re-normalized difference vegetation index	RDVI	$RDVI = \sqrt{NDVI \times DVI}$	Reujean and Breon（1995）

Model simulation

A canopy spectural reflectance data set was input with leaf N content data (from all examined soybean developmental stages) into the EXCEL software to calculate for associated factors across the whole scanning spectrum. The data reflectance data format with highest associated factors was chosen for model construction. The wavelength corresponding to the highest associated factor was chosen as characteristic wavelength to nutrient N. Then, a linear regression model was constructed in EXCEL with the chosen format of data set on the characteristic wavelength. Simulation models for P and K were constructed with the same protocol.

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Software tools

All vegetation indices were calculated with a self-programmed software. All other data processing and analysis were performed with the the EXCEL software.

Results and analysis

Changes of soybean canopy reflectance spectrum at different growth stages

The structure and physiological properties of soybean canopy change as plants grow, and those alterations will affect characteristics of the canopy spectral reflectance. When examining the whole spectral curve in the visible light range of 400~680 nm, it was found that the highest reflectance occurred at the branching stage, it then declined and stabilized at a lower level during seed bulging and maturation stages (*Fig. 1*). This spectral band region is influenced strongly by plant pigments. It is possible that as soybean plants grow, leave accumulate more chlorophyll thus increasing the absorbance capacity for visible light. The same physiological state continues until pod-set stage. Thereafter, plant pigment content gradually declines to a stable low level, thus spectral reflectance rate also stabilizes at a different level. In the near-infrared region (760~1000 nm), the reflectance rate also changed significantly with plant growth. The canopy spectral reflectance was at the highest level at the branching stage. At more mature growth stage, the reflectance rate gradually decreased till reaching the lowest level at the maturation stage (*Fig. 1*). Furthermore, it was also found that from flowering to seed set period, and from seed bulging and maturation period, the spectral reflectance curve had several big fluctuations. It is likely that during the first period from flowering to seed set stages, large amounts of leaf mineral nutrients were transported to seed pods, and then during the second period from seed bulging to maturation stages, plants started to age and deteriorate resulting in physiological functional decline.

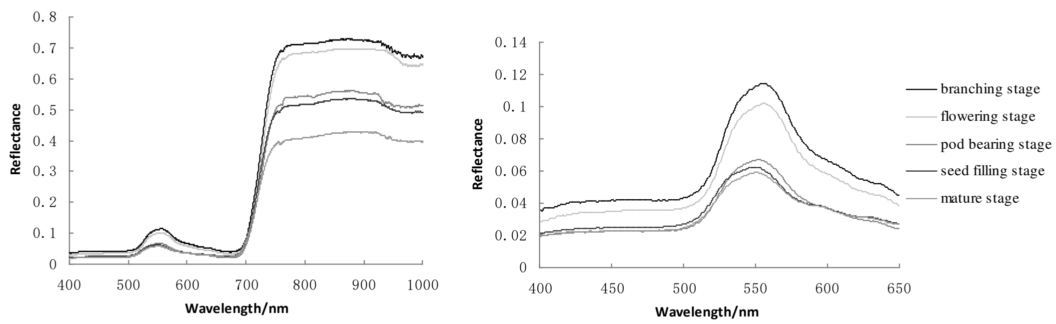

Figure 1. *Hyperspectral reflectance of soybean at different growth stages*

Characteristic changes in soybean canopy total N, P K contents

Changes of soybean leaf mineral nutrients at different growth stages were shown in *Fig. 2*. The total N content decreased as plants grew with a sharp decline from branching to flowering stages then it slowed down at the post-flowering stage. Contents of P and N seem to have positive synergistic effects. The application of P fertilizer treatment had a positive effect on N content. For plants passed the flowering stage, leaf P content was higher for the one that were fed with P fertilizers than those without P

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fertilizers; P-1 treatment had a more pronounced effect on soybean leaf N content compared to P-2 treatment; leaf total P content decreased gradually from branching to flowering stages, and then declined post the flowering stage. The highest leaf P content was found in high-rate P fertilizer treatment which is significantly higher than the non-P-fertilizer control; soybean leaf K content increased gradually from branching to flowering stages and it started to decline post the flowering stage. Plants receiving P fertilizer treatment also had higher K contents compared to control; but there were no big differences among between different levels of P fertilizers. These results indicate that appropriate use of P fertilizer helps to improve leaf K nutrient status.

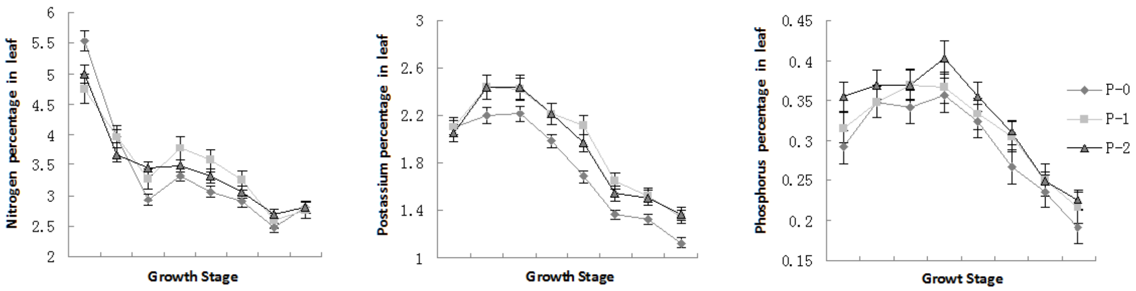

Figure 2. *Variation of total N, P, K content in soybean canopy at different growth stages*

Simulation models for soybean leaf total N, P, K contents

In this study, the original data of canopy spectral reflectance and those transformed by the function of reciprocal, derivative and algorithm, were used to identify the optimal band regions that would produce a good correlation between canopy spectral data and leaf total N, P, K contents, and to establish simulation models of these three mineral nutrients. Results indicate the correlation efficient (r) of the simulation equations depends on data transformation *(Table 2)*. The correlation efficient of the four transformed datasets was at an extremely significant level ($p<0.01$) but from different bands for total N, P and K. The first and second-derivatives of the spectral reflectance had a better correlation with N, P, and K than the other three transformed datasets. These results indicate that the first and second derivatives may be able to eliminate side-effects from soil, the depth of leaf color, and other environmental factors. Thus they are correlated with physiological-chemical properties of chlorophyll and other growth factors (Malthus et al., 1991).

Table 2. *Correlation between soybean leaf essential mineral nutrients and transformed spectral data (N=144)*

Spectral data	Leaf total N (%)		Leaf total P (%)		Leaf total K (%)	
	Bands	Correlation coefficient	Bands	Correlation coefficient	Bands	Correlation coefficient
ρ	569nm	0.5116**	771nm	0.3749**	777nm	0.3749**
1/ρ	566nm	-0.3967**	768nm	-0.4092**	566nm	-0.3967**
Log(ρ)	567nm	0.4798**	769nm	0.3939**	992nm	0.3925**
ρ′	625nm	-0.7720**	909nm	-0.5960**	908nm	-0.5631**
(1/ρ)′	553nm	-0.5203**	870nm	0.5569**	798nm	0.4233**

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Log(ρ) ′	592nm	-0.7003**	909nm	-0.6172**	592nm	-0.5447**
ρ″	423nm	-0.7829**	806nm	-0.5473**	432nm	-0.5398**
(1/ρ)″	519nm	-0.5279**	910nm	-0.5536**	539nm	0.4756**
Log(ρ) ″	423nm	-0.7138**	680nm	-0.5894**	539nm	-0.5461**

Note:** indicates an extremely significant correlation (P< 0.01), * indicates a significant correlation (P< 0.05).

It was shown that soybean leaf total N content has the best and positive correlation with the second-derivative (ρ″) of the spectral reflectance at the 423 nm band (*Table 2*); the simulation equation is: y=-19.153x+3.1114 (r=-0.7829, p<0.01; RE=0.1713); the measured and analog value predicted from the model is r=0.7908 (p<0.01) (*Fig. 3*). The simulation equation was constructed for the total P content and Log(ρ) ′ of the first-derivative of spectral reflectance at 909 nm as: y=-0.2939x+0.5889 (r=-0.6172, p<0.01; RE=0.2428); The measured and simulated values were significantly correlated (r=0.7386, p<0.01) (*Fig. 4*). To determine the optimal correlation coefficient between soybean leaf total K content and the first derivative of spectral reflectance at 908 nm, a function model was constructed using the derivative data and leaf total K content and it is written as: y=-0.4157x+1.874(r=-0.5631, p<0.01; RE=0.1345) and there was a significant correlation between the measured data and the analog values predicted using the model (r=0.6421 (p<0.01) (*Fig. 5*).

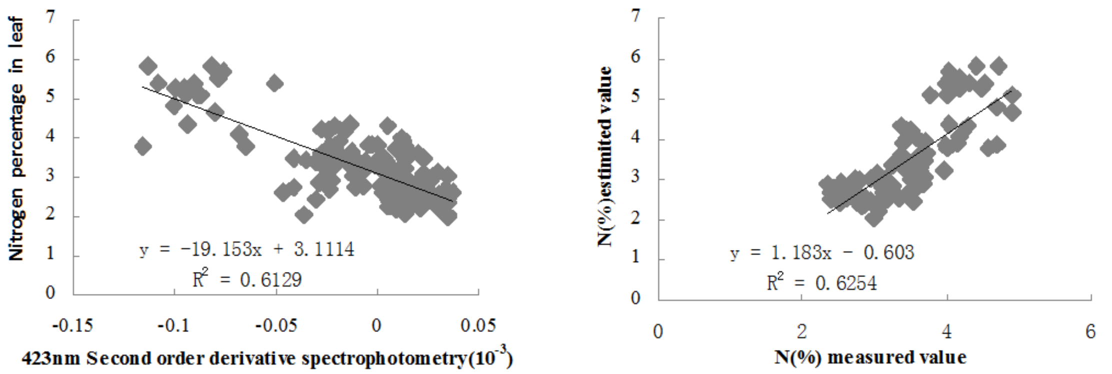

Figure 3. *Correlation analysis between total leaf nitrogen content and optimal transformation of the reflectance spectrum of soybean canopy (N=144)*

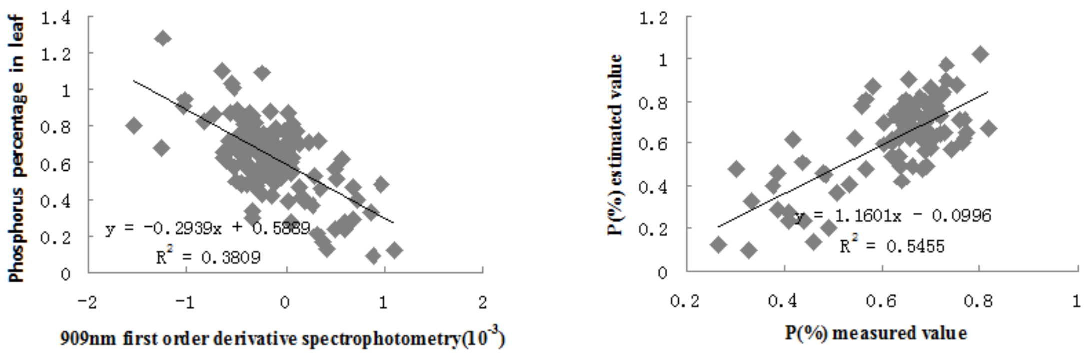

Figure 4. *Correlation analysis between the simulated and the measured total phosphorus content of soybean leaves (N=90)*

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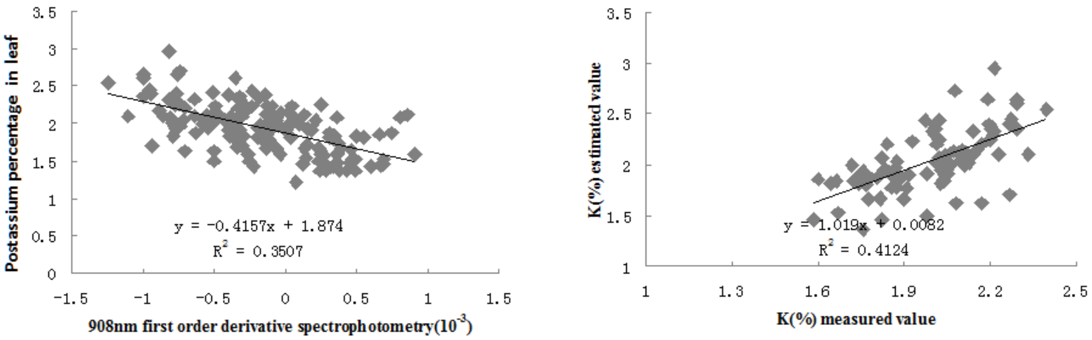

Figure 5. *Correlation analysis between total leaf potassium content and the derivative spectra (N=144) in soybean canopy*

Correlation between red edge parameters and soybean leaf N, P, K contents

Results of the correlation analysis between the red edge parameters including red edge position (λ_{red}), red edge slope ($D\lambda_{red}$) and red edge area (S_{red}) and leaf total N, P, K were contained in *Table 3*. Among the simulation models, the fit equation for red edge area (S_{red}) and soybean leaf total N content is: y=3. 1714x+1.7001(r=0.4676, p<0.01; RE=0.2439). The correlation coefficient between measured and analog values from simulation is: r=0.7088 (p<0.01) (*Fig. 6*). The simulation equation for the red edge slope ($D\lambda_{red}$) and total P content is: y=20.204x+0.3811(r=0.3981, p<0.01; RE=0.2739); the correlation coefficient between the measured and analog values is r=0.5918 (p<0.01) (*Fig. 7*). The simulation equation for the red edge area (S_{red}) and leaf total K is: y=1.009x+1.589 (r=0.4121, p<0.01; RE=0.1506); The correlation coefficient between measured and analog values is r=0.5791 (p<0.01) (*Fig. 8*). These results indicate that red edge area (S_{red}) and the slope ($D\lambda_{red}$) and the position (λ_{red}) each have lower correlation with leaf total N, P and K contents. Therefore these red edge parameters are not suitable for the purpose of predicting leaf total N, P, K contents under the experimental conditions.

Table 3. *Correlation between soybean canopy red edge parameters and leaf nutrient contents*

Red edge parameters	Leaf total N (%)	Leaf total P (%)	Leaf total K (%)
Red edge position λred	0.2582**	0.0964	0.0964
Red edge slope Dλred	0.4590**	0.3981**	0.3608**
Red edge area Sred	0.4674**	0.3641**	0.4120**

Note:** indicates an extremely significant correlation (P< 0.01), * indicates a significant correlation (P< 0.05), (N=144).

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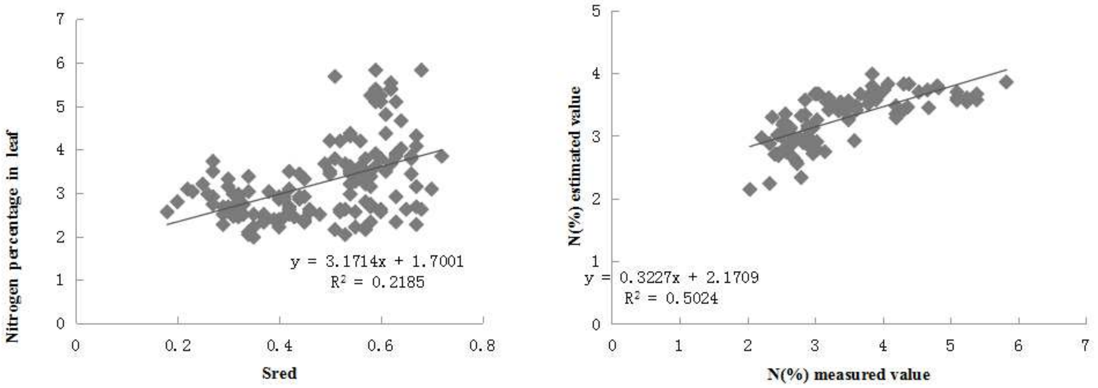

Figure 6. *Correlation analysis between the simulated total nitrogen content and the red edge parameters (N=144)*

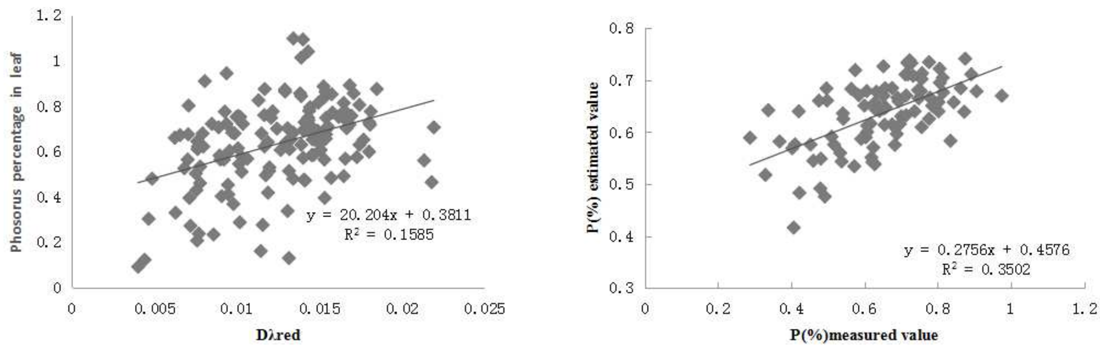

Figure 7. *Correlation analysis between the total leaf phosphorus contents and Dλred and Sred (N=144) of soybean canopy*

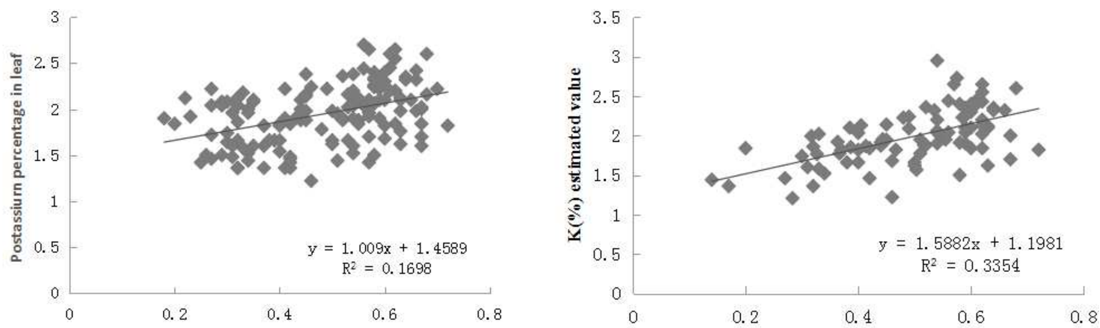

Figure 8. *Correlation analysis between the simulated and measured total potassium content of soybean leaves (N=90)*

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Vegetation index simulation model

Data of spectral reflectance from a selection of optimal bands were used to construct simulation models for vegetation indices and leaf total N, P and P content. The four generally used vegetation indices, DVI, RVI, NDVI and RDVI, were calculated to select the optimal spectral bands (*Table 4*), and then soybean LAI simulation model were constructed for these growth parameters. After simulation test, the equations giving the best correlation coefficient (r) and smallest average relative error (RE) was selected as the optimal simulation model.

Table 4. *Soybean leaf mineral nutrients and vegetation index simulation model analysis (N=144)*

	Vegetation index	Spectral band regions (nm)		The fitted equation	Correlation coefficient	Average relative error (RE)
Total leaf N content (%)	DVI	762	743	y=22.006x+2.0303	0.6242**	0.1195
	RVI	762	743	y =13.044x-11.343	0.5559**	0.1271
	NDVI	762	743	y =29.693x+1.5944	0.5576**	0.1269
	RDVI	762	743	y = 26.604x+1.7856	0.6152**	0.1192
Leaf total P (%)	DVI	771	757	y=16.62x+0.2946	0.5750**	0.2292
	RVI	911	756	y =13.044x-11.343	-0.3887**	0.2709
	NDVI	762	693	y =29.693x+1.5944	0.4207**	0.2667
	RDVI	771	757	y = 15.709x+0.3168	0.4656**	0.2466
Leaf total K content (% in dry weight)	DVI	762	734	y=3.2464x+1.5448	0.4755**	0.1468
	RVI	762	742	y =3.3287x-1.82	0.4207**	0.1534
	NDVI	761	735	y =4.2976x+1.4181	0.4591**	0.1471
	RDVI	762	722	y = 2.3962x+1.2782	0.5318**	0.1402

Note:** indicates an extremely significant correlation (P< 0.01), * indicates a significant correlation (P< 0.05); x is vegetation index

The simulation equation for leaf N content was constructed using the difference vegetation index (DVI) [762,743]. It is described as: y=22.006x+2.0303(r=0.6242, p<0.01; RE=0.1195). The measured data and simulated analog values have a significant correlation (r=0.7678, p<0.01) (*Fig. 9*). The simulation equation for DVI [771,757] and leaf total P is written as: y=16.62x+0.2946(r=0.5750, p<0.01; RE=0.2292), with a significant r (r=0.7572, p<0.01) between the predicted analog values and the measured data (*Fig.10*). The simulation equation using the normalized difference vegetation index (NDVI) [762,722] is: y = 2.3962x+1.2782(r=0.5318, p<0.01; RE=0. 1402). The measured data and simulated analog value has a significant correlation (r=0.6303, p<0.01) (*Fig. 11*).

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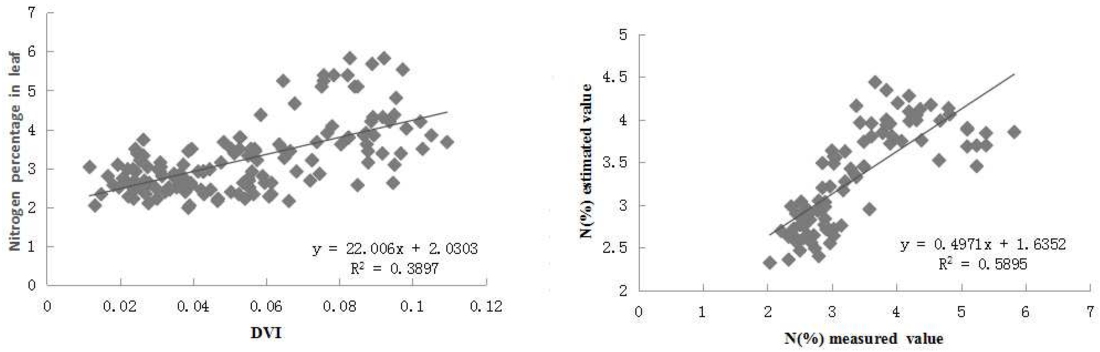

Figure 9. *Correlation analysis between total leaf nitrogen contents and vegetation index of soybean canopy*

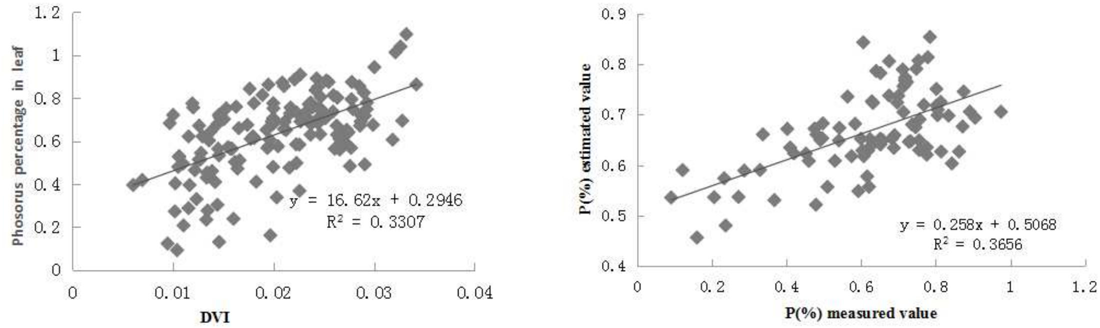

Figure 10. *Correlation analysis between total leaf phosphorus content and vegetation index of soybean canopy*

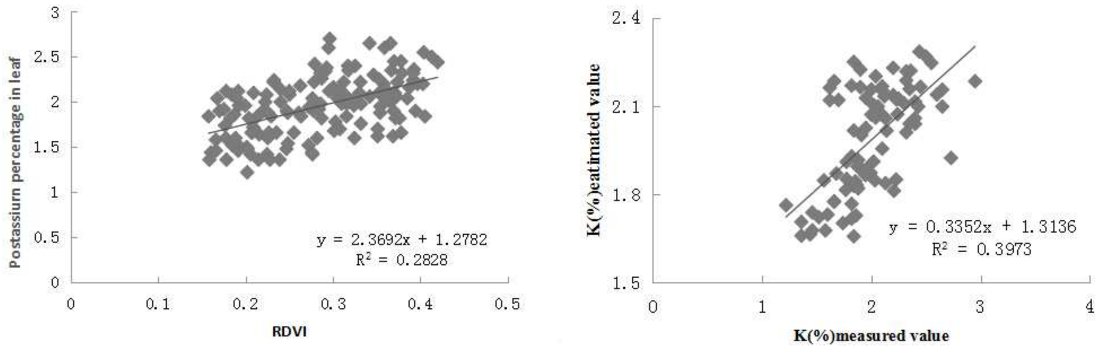

Figure 11. *Correlation analysis between total leaf potassium content and vegetation index of soybean canopy*

Conclusion and discussion

The following conclusions have been *drawn* from this study:

- *Models for hyperspectral simulation* of major mineral nutrient contents (leaf total N, total P, total K) were constructed using the original data and those *transformed* by the *reciprocal* function, logarithm, derivative, of *spectral*

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reflectance, the red edge parameters, and the four vegetation indices *of soybean canopy. It was* found that the first derivative and the second *derivative of spectral reflectance* at 423nm *can reliably predicate leaf total N content using the* following equation: y=-19.153x+3.1114 (r=-0.7829 RE=0.17130). The predicted data using this modes is well- correlated value (r=0.7908**) with measured data, thus the model has competitively high predictive power.

- Among model equations developed using the l*ogarithmic spectrum* data, the first *and* the second *derivatives and* DVI were able to predict leaf total P content at a degree of high accuracy. At the 909nm wavelength, *the equation for* the first *derivative of logarithmic spectrum is:* y=-0.2939x+0.5889(r=-0.6172 RE=0.2428),which produces a significant correlation coefficient (r=0.7386**, p<0.01),between measured and analog values and it is the best among the three models.
- The derivative- transformed and renormalized difference vegetation index (RDVI) can both accurately predict leaf total K content. At 908nm, the equation using the first derivatives of spectral data is: y=-0.4157x+1.874 (r=-0.5631 RE=0.1345). The correlation coefficient between measured and analog values is r=0.6298**, therefore it is the most powerful equation for modeling leaf K content.
- In summary, in this study, the reflectance spectral data from soybean canopy were collected and processed to develop simulation models for leaf growth parameters and mineral nutrient contents. A wide application of these models is expected in crop management. An extremely strong correlation was found between content of major nutrients (total N, P K) and the transformed data of canopy spectral reflectance. The sensitive bands for total leaf N, P K contents are 423nm, 909nm and 908nm. Several highly accurate simulation equations were developed for these bands. But during the process of screening for the optimal band range, it was found that a large number of bands had an extremely significant correlation (p<0.01) with leaf mineral contents, furthermore, the differences between those bands were very small.
- The hyperspectral features within the visible light region (400~700nm) are sensitive to N status in the canopy of cotton (Wang and Li, 2012). The 350~730 nm and 1420~1800 nm are the wavelength ranges that are sensitive to P contents in maize plants (Wang et al., 2007). These researches consistently have demonstrated that there is no single band that can produce enough spectral data to simulate a plant growth parameter, instead, data from "a range of optimal wavelengths" have to be used to develop the spectral signatures. More studies will be conducted to establish and validate the most stable and most effective wavelengths for each of the plant growth parameters for soybean crop.

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OPTIMIZATION OF RED BEAN SEEDS ULTRASONICATION FOR INCREASING GERMINATION AND SEEDLING GROWTH, USING ARTIFICAL NEURAL NETWORK

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Abstract. In most cases, there is no positive strong relation (correlation > +0.95) between germination percentage (G) and individual seedling dry weight (W) over treatment levels, which indicates the different response of GW to treatment. In such cases, it is difficult to choose/recommend a treatment level as a best one. Artificial neural network-based optimization (interpolation of treatment levels for possible simultaneous maximum increase in both GW) is the solution to such difficulties. This study aimed at optimizing ultrasonication (pre-soaking duration, temperature and duration of irradiation) of *Phaseolus vulgaris* L. seeds. Treatments were factorial arrangement of 5 pre-soaking durations (2, 4, 6, 8 and 12 hours), 5 irradiation durations (0, 3, 6, 9 and 12 minutes), and 4 irradiation temperatures (temperature of ultrasound device:17, 22, 27, and 32 ^{o}C). The results indicated that the structure 3:4:4:2 of neural network appeared to be appropriate for predicting GW responses. This structure showed higher R^2 in both learning and test phases, when the model was based on Secant Hyperbolic function. The optimized combination of treatments was irradiation temperature of 26.99 ^{o}C, irradiation duration of 5.91 minutes, and pre-soaking duration of 3.20 hours.
Keywords: *pre-soaking duration, irradiation temperature, irradiation duration, seedling dry weight, prediction.*

Introduction

Several methods have been used for seed pre-sowing treatment in order to increase the germination and uniformity i.e. gibberellic acid, sulphuric acid, and hot water treatment (Machikowa et al., 2013). However, seeds treated by these methods have to be rapidly grown and cannot be storaged. Therefore, the methods that increase seed germination but do not affect their viability during the storage are much needed. Applications of ultrasound have been used to induce faster and greater seed germination of the Norway spruce, barley, orchids and other crops. It has been reported that the optimum condition of treated seeds lead to uniform and rapid germination (Machikowa et al., 2013). Ultrasonication not only promotes germination rate (Gordon et al., 1963; Shimomura, 1990; Keshvari et al., 2008; Yaldagard et al., 2008), but also accelerates water uptake and seed coat permeability via creating temperature and mechanical effects on cell membrane and hence promotes germination (Yıldırım and Mehmet, 2015; Gavrilo et al., 1996).

Ultrasonic waves have various applications ranging from seed treatment and elimination of pests and pathogens to genetic engineering and gene transfer (Yaldagard et al., 2008). The first research on the field of biological effects of ultrasound was reported by Harvey and Loomis (1928). In a research conducted on seed germination of

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Assa foetida L., the 0, 4, 8 and 12 minutes ultrasonication resulted in germination rates of 5, 35, 57.75 and 62.5 day^{-1}, respectively; in this study, the 4-minute irradiation was selected as the best treatment (Abdali et al., 2010). Machikowa et al. (2013) reported that seed irradiation with ultrasound waves enhances seedling vigor; this positive effect of ultrasound on seed germination results from change in cellulosic membrane and enhancement of nutrient uptake and transfer to inside of seed and growing seedling. Faryabi et al. (2008) studied effects of ultrasound waves on physiological and morphological processes of capsicum pepper (*Capsicum annuum*) and radish (*Rhaphanus sativus*); in their experiment, the seeds were treated under ultrasound waves of 42 KHz for 0, 4, 6 and 8 minutes at 25 ^{o}C under cool light; results indicated that the best treatment for radish was sonication for 6 minutes.

It should be noted that the amplitude of ultrasound plays critical role in activation/inactivation of enzymes. Many reports confirm enhancement of enzymatic activity of free enzymes under slight radiation of ultrasound; for example, activity of alpha-chymotrypsin on casein is enhanced under slight amplitude of ultrasound; while the activity of this enzyme is reduced at higher amplitudes (Ishimori et al., 1981).

Artificial neural network is a data analysis system inspired from human brain that analyzes the data using a large number of small processors. These processors are arranged as a consistent network and act in parallel to solve a problem. In such networks, a data structure is designed to act like a neuron. An artificial neuron is system which consists of many inputs and one or a few outputs. The network includes layers and weights components. Network behavior depends on the interrelations among the members. In general, there are three types of neuron layers in neural networks:

- Input layer (regressor): reception of raw material (R_1, …, R_n) entering the network.
- Hidden layer(s): performance of these layers is determined by inputs and the weights of their relations (Z_1, …, Z_2). The weights between input and hidden units determine when a hidden unit should be active.
- Output layer: function of output layer depends on function of hidden unit and the weight of relation between hidden and output layer (Y_1, …, Y_n).

Neural networks have many applications such as prediction of continuous variables such as soil moisture (Chang and Islam, 2000), sampling (Zhang and Barrion, 2006), estimation of grain biomass and yield (Drummond et al., 2003). Using neural network, Gholipoor et al. (2013) optimized the traits affecting barley seed and suggested that genetic improvement of barley seeds based on the optimized seeds can significantly improve seed yield. By optimization of mineral concentration in sugar beet tuber using artificial neural network, the optimal concentration of calcium, magnesium, nitrogen, potassium and sodium was estimated as 0.37%, 0.35%, 0.97%, 4.67 mili equivalent/100 g, and 0.33%, respectively. Under optimized concentration of minerals, potential yield of tuber and sugar was enhanced by about 17% (Gholipoor, 2012).

To the best of our knowledge, there has been no report about possible interaction of pre-soaking duration and ultrasonication temperature with ultrasonication duration on seedling growth and germination percentage of red bean. Regarding these issues, the main objective of this study was to find the best ultrasonication duration, pre-soaking duration and ultrasonication temperature to have highest germination percentage and seedling growth.

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Due to the fact that in most cases, there is no positive strong relation (correlation > +0.95) between germination percentage and individual seedling dry weight over treatment levels (Golipoor et al., not published), these traits tend to have different response to a treatment. In such cases, it is difficult to choose/recommend a treatment level as a best one. Artificial neural network-based optimization (interpolation of treatment levels for possible simultaneous maximum increase in both germination percentage and individual seedling dry weight), which was used here, is the solution to such difficulties.

Materials and Methods

Laboratory experiment

An experiment, as completely randomized design with three replications, was carried out on red bean (*Phaseolus vulgaris* L.) seeds in Seed Technology Center of Shahrood University of Technology in 2015. Treatments were factorial arrangement of five pre-soaking durations (2, 4, 6, 8 and 12 hours), five ultrasonication durations {0 (control), 3, 6, 9 and 12 minutes}, and four ultrasonication temperatures (temperature of ultrasound device: 17, 22, 27, and 32 ^{o}C). The ultrasonic bath (digital ultrasonic, Model 4820-CD) with constant frequency of 24 kHz of ultrasonic waves was used for irradiating the seeds.

Twenty five seeds were selected for each petri dish. The germinated seedlings were counted on daily basis until it was found no change in number of germinated seedlings for two consecutive days. The germinated seedlings were multiplied by four to get the germination percent. The seedlings were separated form seeds and dried in oven for 48 hours at 70 ^{o}C. Then they were weighed. The individual seedling dry weight and germination percentage were subjected to analysis of variance, using the SAS software (version 9.1).

Optimization of regressors using artificial neural network

Data of input (regressors or independent variables; pre-soaking duration, ultrasonication duration, and ultrasonication temperature) and output variables (dependent variables; individual seedling dry weight and germination percentage) were first arranged consecutively and divided into two learning (70%) and test (30%) parts. Then, they were standardized using the following formula (Rohani et al., 2011):

$$Y_i = 0.8 \times \frac{X_i - X_{min}}{X_{max} - X_{min}} + 0.1 \quad \text{(Eq.1)}$$

where, Y_i represents standardized data, X_i denotes non-standardized data, X_{min} stands for the smallest data and X_{max} denotes the largest data. Using this formula, the input data are placed between 0.1 and 0.9. Perceptron multilayer neural network was used in this research. Using QNET software, back propagation algorithm was used for network learning. Various numbers of hidden layers and following four activation (transfer) functions were tested:

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- Sigmoid function:

$$f\left(\sum W_{ij}X_i\right) = \frac{1}{1+e^{-\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)}} \quad \text{(Eq. 2)}$$

- Hyperbolic tangent function:

$$f\left(\sum W_{ij}X_i\right) = \frac{e^{\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)} - e^{-\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)}}{e^{\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)} + e^{-\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)}} \quad \text{(Eq. 3)}$$

- Hyperbolic secant function:

$$f\left(\sum W_{ij}X_i\right) = \frac{2e^{\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)}}{e^{2\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)} + 1} \quad \text{(Eq. 4)}$$

- Gaussian function:

$$f\left(\sum W_{ij}X_i\right) = \frac{1}{\sqrt{2\Pi\sigma}} e^{\frac{-\left[\left(\sum_{i=1;j=1}^{n} W_{ij}X_i\right)-\Pi\right]^2}{2\sigma^2}} \quad \text{(Eq. 5)}$$

where W is weight, X regressor, σ standard error, and Π pi value.

For evaluation of efficacy of perceptron neural network in predicting variation of seedling dry weight and germination percentage, the correlation coefficient (r), mean of absolute error (MAE) (eqation 6), root-mean-square error (RMSE) (eqation 7) and relative standard deviation (RSE) (eqation 8) were used:

$$\text{MAE}=\frac{1}{n}\sum_{i=1}^{n}\left|Y_i - \hat{Y}_i\right| \quad \text{(Eq. 6)}$$

$$\text{RMSE}=\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2} \quad \text{(Eq. 7)}$$

$$\text{RSE}=\frac{\sqrt{\frac{1}{n}\sum_{i=1}^{n}\left(Y_i - \hat{Y}_i\right)^2}}{Ybar} \quad \text{(Eq. 8)}$$

where, Y_i stands for observed output, Ybar denotes mean of observed output, $\hat{Y}_i$ is estimated output and Ybar stands for mean of observed output. Optimization of the regressors was performed through following three steps (Gholipoor et al., 2012), using QNET software:

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Step 1

Out of the regressors, a regressor (irradiation temperature) with strong relation with seedling dry weight and germination percentage was put in the model as the output. Seedling dry weight and germination percentage were considered as model inputs. Data were randomly arranged in line and divided in to two learning (70%) and (30%) parts. Different scenarios were tested and the superior model was selected for each output variable. Based on the superior model, irradiation temperatures were estimated. Each of these estimated values can be potentially the optimal temperature of irradiation for achieving maximum seedling dry weight and germination percentage. The temperature values within tested temperature range were selected (*Table 1*).

Step 2

Using original and estimated data in previous step, other regressors were individually put in the model as output. Model inputs include seedling dry weight, germination percentage and irradiation temperature. Like previous step, data were divided into two groups, different scenarios were tested and superior model was selected. Using this superior model, output was estimated. The values within the tested values range were selected (*Table 1*).

Step 3

For prediction of seedling dry weight and germination percentage using estimated regressors, the neural network model developed earlier was used. The predicted values of seedling dry weight and germination percentage were presented in *Table 1*.

Table 1. *The values of input layer and output layer obtained during 3 steps of optimization*

Irradiation temperature (°C)	Pre-soaking duration (hour)	Irradiation duration (minute)	Seedling dry weight (mg.seedling^{-1})	Germination percentage
17.09954	2.10646	0.24924	0.21596	87.60438
17.02344	5.86315	0.32141	0.28215	85.12283
27.06161	7.84034	5.74945	0.26376	88.31532
26.40762	3.82008	4.06223	0.30046	85.75182
27.31862	5.57029	6.15325	0.27945	84.88587
26.47268	5.96725	5.54694	0.28916	87.97795
31.98362	9.14480	11.98689	0.32757	87.49119
16.67356	12.13849	8.54124	0.32846	83.72129
32.13457	3.00300	11.52284	0.25481	98.56429
26.52020	12.56619	6.83293	0.27769	96.19994
30.24704	10.97450	3.64731	0.34973	93.71431
17.30544	12.35777	0.02311	0.37052	78.55254
29.81815	3.94204	1.51304	0.24119	90.53204
21.94637	11.87978	6.04494	0.36646	88.20433
26.65126	6.11294	9.39194	0.35170	94.77507
26.99524	3.20238	5.91580	0.48892	100.00000
17.05237	3.03896	11.89409	0.22875	78.82457

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26.44926	7.01220	5.37727	0.27758	90.83411
16.87708	8.02893	6.13390	0.35306	88.75356
28.66749	6.01602	10.52825	0.32855	91.69682
26.89883	2.19398	11.62910	0.26829	93.83383
29.31988	1.93755	0.00086	0.25591	84.39989
32.09550	2.70404	9.50710	0.28553	96.12952
25.93219	6.18928	0.17911	0.18947	80.17261
22.18381	1.48999	3.35011	0.31006	97.18890
26.45018	7.03788	5.35032	0.27777	90.95387
21.63031	8.07121	11.65102	0.28035	89.79551
17.17671	12.53354	6.24890	0.31022	83.34408
21.99522	6.00882	4.94837	0.27886	97.32630
22.16796	7.50277	0.30533	0.27186	88.85596
26.79841	3.27539	6.09689	0.31378	93.06115
26.54209	7.88851	7.02443	0.19549	80.10416
31.83300	12.26652	3.68888	0.36477	95.79995
26.54753	6.31874	7.11125	0.21100	80.38619
31.96131	10.17290	11.87890	0.31806	86.72826
22.01154	8.00452	8.61893	0.36592	93.77309
26.90156	2.60097	5.81457	0.31348	95.63342

Results and Discussion

The substantial differences in treatments produced a wide range of variation in the values of the output layer (*Table 2*). For instance, the maximum value of seedling dry weight was almost twice of minimum value; such variations are necessary for successful learning and predicting processes of neural network. Specially, when there is no strong relation ($r>+0.95$) between output variables; the relation of seedling dry weight with germination percentage was 0.34 which confirms the different response of these traits to treatments.

The result of analysis of variance indicated that in addition to simple effects, the double and triple interactive effects of factors pre-soaking duration, temperature regime, and irradiation duration were significant on both seedling dry weight and germination percentage traits (data not shown). This shows that the effect of one factor on these traits tends to be changed with changing the level of other two factors. Such interactive effects, on one hand, and not significant relation between traits, on the other hand, indicate the complicate relation between regressors and output variables. Neural network-based optimization is capable of solving such multi-response complicate problems.

The performance of the MLP tended to improve as the number of hidden neurons increased. However, too many neurons in the hidden layer caused over-fitting problems. This situation allowed good network learning and data memorization but also produced a lack of any ability to generalize. However, the network was unable to learn if a small number of neurons were used in the hidden layer. Usually the number of layers and neurons nodes of hidden layer (s) is typically determined by trial-and-error. The best structure of neural network was 3:4:4:2.

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Statistical indices were used for evaluation of neural network models for (1) different activation functions, (2) number of hidden layers, and (3) number of neurons in each hidden layer. Combination of these factors can generate more than 50 scenarios and presenting statistical indices for all of these scenarios provides an unnecessary and bulky table. Therefore, after selection of the best number of hidden layer and neuron number in the hidden layer, only statistical indices for activation functions are presented (*Table 2*). According to these indices, higher accuracy of a neural network is obtained when the model not only has higher coefficient of determination, but also has lower absolute error mean, lower relative standard deviation, and lower root-mean-square error. This is the case for hyperbolic tangent activation function as is evident in *Table 3* and *Figs. 1 and 2*.

Table 2. *Statistical properties of input and output layers of neural network model*

Input+output	Range	Mean	Maximum	minimum
Irradiation temperature (°C)	15.0000	24.5758	32.0000	17.0000
Pre-soaking duration (hour)	10.0000	6.3434	12.0000	2.0000
Irradiation duration (minute)	12.0000	6.0303	12.0000	0.0000
Seedling dry weight (mg. seedling^{-1})	0.2601	0.2966	0.4056	0.1905
Germination percentage	23.3333	90.2862	100.0000	76.6667

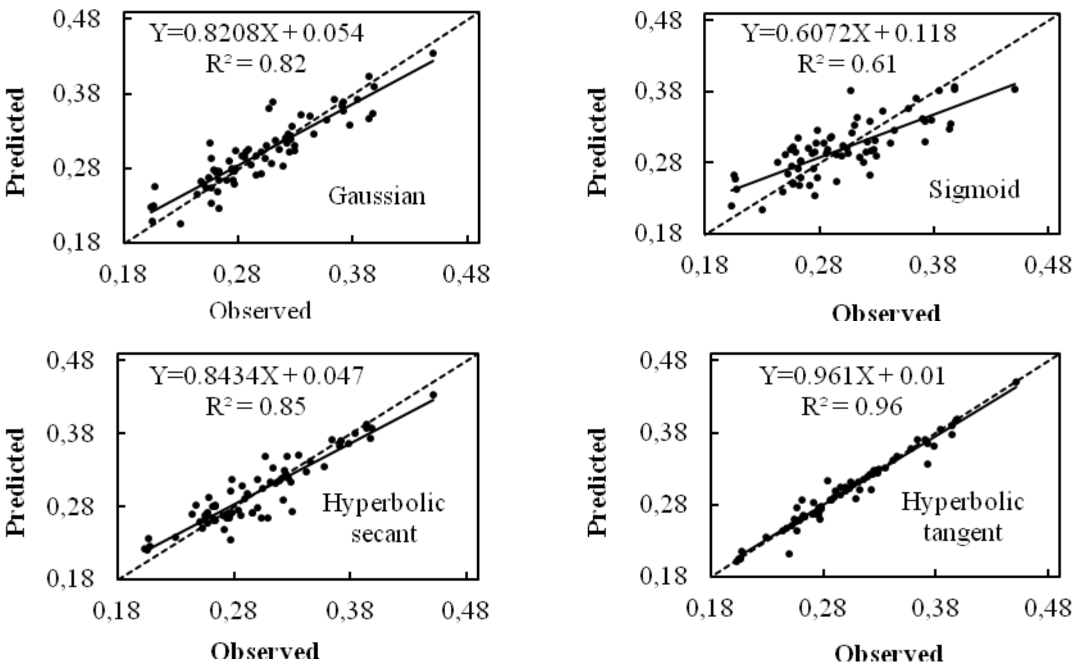

Figure 1. *Sensitivity analysis of neural network using four activation functions for prediction of seedling dry weight of red bean in learning phase*

Optimization results indicated that the highest germination percentage and seedling growth optimized by neural network were 100% and 0.48892 g, respectively (*Table 1*), which is about 19% higher than the observed maximum dry weight of seedling. These values were obtained under treatment with irradiation temperature of 26.99 °C,

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irradiation duration of 5.91 minutes and pre-soaking for 3.20 hours. This combination resulting from neural network is different from treatment combination in routine analyses (temperature of 32 ^oC, pre-soaking for 12 hours and irradiation duration of 12 minutes).

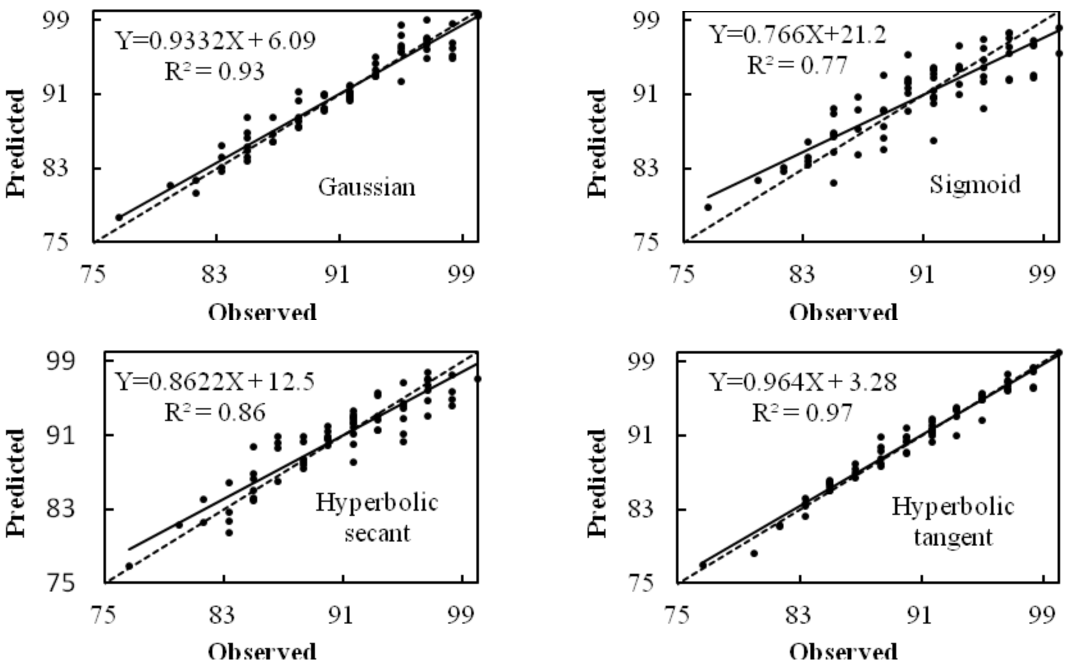

Figure 2. *Sensitivity analysis of neural network using four activation functions for prediction of germination percentage of red bean in learning phase*

The best irradiation level obtained here for *Phaseolus vulgaris* L. , 5.91 minutes, is similar to 6 minues for *Capsicum annuum* L. and *Rhaphanus sativus* L. (Faryabi et al., 2008), but inconsistent with 4 minutes for *Assa foetida* L. (Abdali et al., 2010). Such differences or similarities imply the expectedly direct relation between hardness of seed coat and exposure duration; in another words, the exposure duration is highly dependent on texture of seed covers. This is because of the fact that the machanical impact of ultrasound is higher than other priming methods like hydro priming, hormon priming, and magnetic field (Majd et al., 2010), as ultrasonication could fragment the seed shell and produce larger porosity on the surface of seeds by captivation of ultrasound (shock waves). Shell fragmentation and enlargement of the pore size of seeds lead to more water retention capacity in dry grains and result in better hydration (Yaldagard et al., 2008).

Table 3. *Statistical indices of various transfer functions*

Transfer function	Dependent variable	RMSE	MAE	RSE	r
Gaussian	Seedling dry weight	0.00567	0.01645	0.01889	0.90550
	Germination percentage	0.04410	1.06378	0.00049	0.96424
Sigmoid	Seedling dry weight	0.00788	0.02642	0.02626	0.78187
	Germination percentage	0.14344	2.03956	0.00158	0.87762
Hyperbolic tangent	Seedling dry weight	0.00191	0.00570	0.00636	0.98012
	Germination percentage	0.11125	0.67271	0.00123	0.98508
Hyperbolic secant	Seedling dry weight	0.00013	0.01521	0.00045	0.92348
	Germination percentage	0.04628	1.55737	0.00051	0.92583

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Regarding contribution of regressors in explaining variation of seedling dry weight and germination percentage, irradiation temperature, irradiation duration and pre-soaking duration have the higher priority, respectively; however their difference was not considerable (*Fig. 3*). This result indicates importance of all the three factors and implies that ignoring one factor may result in unfavorable result. This is an exact and unique property of neural network for optimization (Kashi et al., 2013; Huang et al., 2010; Green et al., 2007; Park et al., 2005; Kaul et al., 2005). There is no published report about relative importance of these factors. Regarding different response of plant species to irradiation it is necessary to study impressionability of other species from these factors.

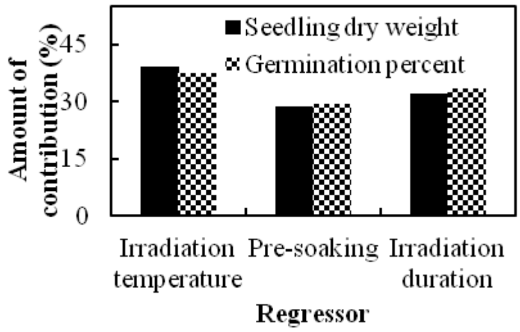

Figure 3. *Contribution of regressors in explaining variation of seedling dry weight and germination percentage*

If ultrasonication is used at optimal conditions (temperature and pre-soaking duration), it will provide positive effects. Irradiation effects result from cavitation phenomenon which causes alteration in cell membrane (Bommannan et al., 1992). One of the reasons for positive effect of ultrasonication on seedling dry weight might be due to reduction in soaking time, as it has been reported for chickpeas (Yildirim et al., 2010), sorghum (Patero and Augusto, 2014) and navy beans (Ghafoor et al., 2015). This improvement has been attributed to a greater reduction of internal resistance than external resistance (Cunningham et al., 2008), as well as possible changes in microstructure by cavitation (micro-channel formation) and/or the so called "sponge effect", causing inertial flow (Patero and Augusto, 2014). The enhanced movement of liquid medium, increase of mass transfer among the organelles within cells and enhanced rate of biochemical reactions are also regarded as the positive effects of irradiation (Bar, 1998).

Conclusion

The results indicated that seedling dry weight and germination percentage tend to differ in terms of response to treatment level (combination of temperature regime, pre-soaking and irradiation duration), as their correlation was not significant. Therefore, selecting each level of treatment does not necessarily increase both traits. Therefore, interpolation of treatment level for possible simultaneous increase in both seedling dry weight and germination percentage, say optimization, was carried out to solve the problems. The optimized values of treatment levels (ultrasonication

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components) were temperature of 26.99 °C, irradiation duration of 5.91 minutes and pre-soaking for 3.20 hours. This procedure could be used in all agricultural researches in which the multi-response investigations, like response of grain yield and grain protein content, are common.

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ISOLATION, MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF BORON-TOLERANT BACTERIAL STRAINS FROM SEWAGE TREATMENT POND OF ISLAMABAD, PAKISTAN

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Abstract. Boron-tolerant bacteria fall in the category of extremophilic organisms as they have the ability to survive in high boron environments. Such bacterial species needs to be characterized to identify new extremophilic organisms from the ecology for biotechnological benefits. In this study, five boron-tolerant bacterial strains, designated as NCCP-132, NCCP-133, NCCP-134, NCCP-135 and NCCP-136 were isolated from sewage sludge treatment pond of Islamabad, Pakistan. These strains grew on media containing 200 to more than 450 mM of boron concentration. However, the microbial growth is found high at low boron concentration. The isolated boron-tolerant bacterial strains were either extremely boron-tolerant or moderately boron-tolerant. The 16S rRNA gene sequences and phylogenetic analyses delineated that all strains were found to be closely related to species belonging to different genera: *Bacillus, Oceanobacillus* and *Lentibacillus*. Strains NCCP-132, NCCP-134 and NCCP-135 are found to be novel species, while NCCP-133 and NCCP-136 are revealed to be previously identified bacterial species. Morphological, physiological and biochemical characteristics of these strains were studied at their optimal growth conditions. Our study inferred that the sewage treatment pond of Islamabad, Pakistan is rich in boron-tolerant extremophilic bacterial population with diverse bacterial communities having a potential to be utilized in various biotechnological applications in future.
Keywords: *boron, 16S rRNA, phylogeny, Bacillus, Lentibacillus, Oceanobacillus*

Introduction

Boron is concentrated in the earth's crust in the form of borate minerals. The largest known boron deposits have been found in Turkey (Col and Col, 2003). Microbes, plants and animals require boron as micronutrient for their growth (Stanier et al., 1966; Goldbach et al., 2010), but the level of required concentration varies among different organisms (Saleem et al., 2011). Boron in lesser concentration has been declared useful for the treatment of candidal vulvovaginitis (Swate and Weed, 1974; Otero et al., 2002), food preservation against different microbes (Nielsen, 2004), and an insecticide against cockroaches (Cochran, 1995). Although boron is an essential micronutrient but a very

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narrow range exists between its sufficiency and toxicity (Nable et al., 1997). Boron toxicity to microbes, plants and animals usually cause growth retardation and deleterious effects on reproductive functions (Col and Col, 2003). In contrary, boron essentiality has been considered good for growing specific types of bacteria such as *Bacillus boroniphilus* (Ahmed et al., 2007a).

Extremophilic organisms possess great capacity to inhabit hostile conditions of salinity, drought, temperature, radiation, etc. (Rampelotto, 2010). During the last decade, boron toxicity has been studied as a new frontier in extremophiles and several novel species have been reported as tolerant to toxic concentrations of boron (Ahmed et al., 2007a, 2007b, 2007c, 2007d). Few bacterial species like *Bacillus boroniphilus, Gracilibacillus boraciitolerans, Chimaereicella boritolerans, Lysinibacillus boronitoleran, Variovorax boronicumulans, Lysinibacillus parviboronicapiens*, have shown various levels of boron tolerance (Ahmed et al., 2007a, 2007b, 2007c, 2007d; Miwa et al., 2008, 2009). *Bacillus boroniphilus* can tolerate >450 mM of boron but also it is reported to require boron for its normal growth. Some strains are reported to accomulate boron in their cells (Miwa et al., 2008, 2009) and found to be comparatively low in boron-tolerance. Boron-tolerant bacterial species maintain lower boron concentration in cells by an efflux mechanism (Ahmed and Fujiwara, 2010). These findings can be exploited for their applications in biotechnology.

Boron-tolerant bacteria needs to be explored in different ecologies worldwide, such as in salt mines and sewage treatment ponds. The objective of current study was to isolate and identify novel boron-tolerant bacterial strains from Pakistani ecology. These studies comprised of isolation and identification of boron-tolerant bacteria using 16S rRNA gene sequencing, and their phenotypic characteristics including morphological, physiological and biochemical characterization. The findings of these studies may provide useful information for basic and applied sciences under extreme conditions of elemental boron toxicity.

Materials and methods

Sample Collection

Samples for isolation of boron-tolerant bacterial strains were collected in sterile bottles from sewage treatment pond of Sector I-9/1, Islamabad, Pakistan (location is shown in *Figure 1*) and then stored at 4°C until isolation of strains.

Isolation and Enrichment

Procedures of isolation and enrichment of cultures were performed to recover boron-tolerant bacterial strains on tryptic soy agar (TSA, Difco^{TM}) medium. Serial dilution of samples was carried out in phosphate buffer saline (PBS) in an appropriate volume. The diluted samples were streaked on TSA medium containing 50 mM boron and incubated at 28 °C for 2-3 days. Isolated strains were sub-cultured many times under similar conditions. Later on, the pure cultures of morphologically different bacteria were obtained and used for further characterization. All of isolated strains were preserved at 4 °C, and also in glycerol (35 % w/v) stocks at -80 °C.

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Growth Optimization

Growth conditions of isolated strains were evaluated at different pH (4.0-10.0), temperature (4-50 °C), and NaCl [0-16 % (v/v)] by allowing bacterial strains to grow in a shaking incubator at 28 °C for 2-5 days. TSB medium was used for optimization of growth conditions at OD_{600}.

Morphological Observation

Morphological observations and comparisons were made using microbiological standard. Bergey's manual was used to characterize pure bacterial colonies on the basis of color, shape, margin, elevation, texture, size, etc. Gram staining and visualization of colony morphology of pure bacterial strains was done by light microscope (Nikon E600, Japan).

Boron-tolerance Assay

Boron-tolerance of each strain was performed by growing the bacterial strains at 28 °C in a shaking incubator using tryptic soy broth (TSB; Bacto^{TM}) medium containing various concentrations of boron from 0-450 mM. Bacterial growth was evaluated using spectrophotometer at 600 nm (OD_{600}) for 3-7 days.

Identification using 16S rRNA Gene Sequencing

Genomic DNA extraction was performed following Ahmed et al. (2007a). It involved suspension of 2-3 well isolated bacterial colonies in 20 µL of Tris EDTA (TE) buffer in a micro-PCR tube. After heating the cells at 95 °C for 10 min, centrifugation was carried out at 6000 rpm for 2-3 min. The pellet was discarded and the supernatant obtained was used as a template DNA for amplification of 16S rRNA gene. Polymerase chain reaction (PCR) was performed using 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') primers and PreMix ExTaq (Takara, Japan) to amplify the 16S rRNA gene of the isolated strains according to previous protocol (Ahmed et al., 2007a) using ABI Veriti PCR Machine (Applied Biosystems, USA) with the optimized PCR program, i.e., initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1:30 min, and final extension at 72 °C for 5 min. Amplification of 16S rRNA gene was confirmed on 0.8% agarose gel. Documentation system (UVIPro Platinum, England) was used for viewing the DNA image. The purification and sequencing of amplified PCR products was performed using 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3') primers and commercial service of Macrogen Inc. Korea (www.dna.macrogen.com).

Phylogenetic Analysis

Sequences were aligned using Clustal X software and the fragment sequences of 16S rRNA gene were assembled by BioEdit. Later on, 16S rRNA gene sequences were submitted to DDBJ (http://www.ddbj.nig.ac.jp/) (*Table 1*). Bacterial strains were identified by BLAST search using 16S rRNA gene sequences. Ez-Taxon server was used to retrieve the sequences of closely related type strains in order to construct phylogenetic trees. MEGA software Version 7 was used for phylogenetic and molecular evolutionary analyses.

Biochemical Characterization

Biochemical analysis of the isolated strains was performed using API ZYM, API 20E and API 50CH (bioMérieux, France) according to the manufactured protocols, whereas oxidase and catalase tests were performed according to the procedure of Cowan and Steel (2004). The suspension medium of API was utilized for inoculation of strips in all tests. API ZYM test was conducted for elucidation of enzymatic activities, and API 50CH strips were used for carbohydrate utilization and other biochemical analysis.

Results and Discussion

Isolation and Identification of Boron-tolerant Bacteria

The bacterial strains isolated from sewage treatment pond (shown in *Figure 1*) were found extremely and moderately boron-tolerant (tolerated up to 450 mM of boron). This site was selected for sampling because it is reported that sewage treatment ponds are rich in bacteria that can grow in environment having high concentrations of boron element. A total of five bacterial strains were isolated because it is recommended that the number of strains studied for each taxon should be at least five, and ideally ten or more; observations on a minimum of three strains are necessary to have some indication of natural variability (Logan et al., 2009). This difference in strains could be due to the environmental changes occurring in different sites of the sewage treatment pond.

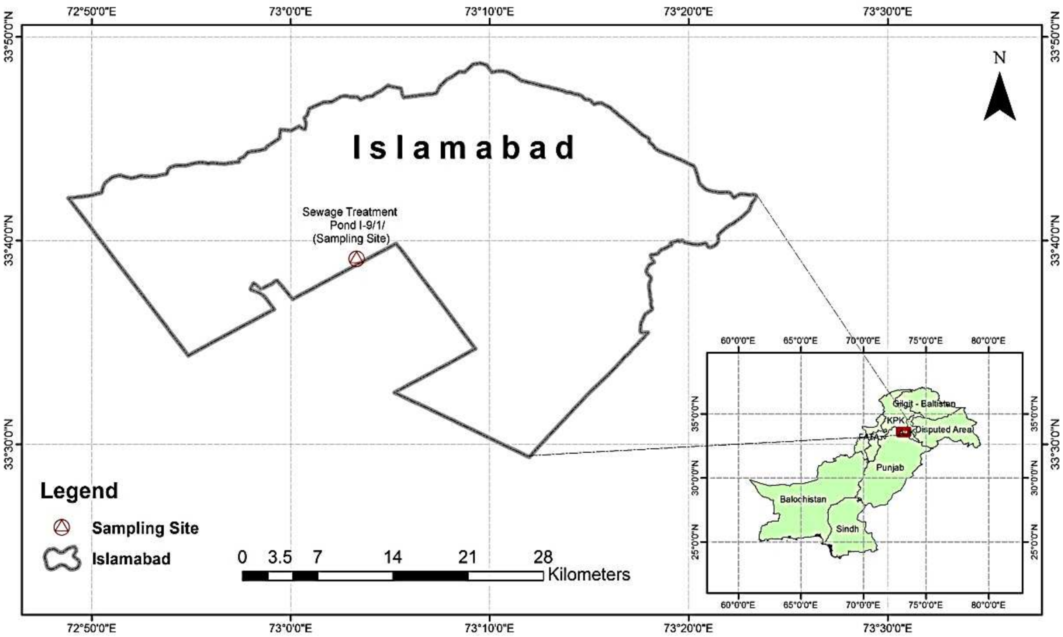

Figure 1. *Map of Pakistan showing sampling site of Islamabad.*

Taxonomic identification of five bacterial isolates was conducted by using the robust method of 16S rRNA gene sequencing (*Table 1*). Thereafter, the phylogenetic position of each strain was determined by the construction of phylogenetic trees (*Figures 1 and 2*). Phylogenetic analysis delineated that the isolated strains belonged to *Virgibacillus*, *Oceanobacillus,* and *Bacillus* (*Table 1*). Two out of five strains (belonging to genus

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Bacillus) shared > 97 % identity with their closest phylogenetic relatives. These result showed that NCCP-133 and NCCP-136 may belong to the known species of *Bacillus oceanisediminis* H2^T (GQ292772) and *Bacillus oryzaecorticis* R1^T (KF548480), respectively, whereas the other three strains (belonging to genera *Bacillus, Virgibacillus* and *Oceanobacillus*) sharing < 97 % sequence similarity confirmed that these strain belong to new species, however further taxonomic characterization experiments need to be performed to delineate these strains as novel species.

Table 1. *Identification of isolated boron-tolerant bacterial strains based on 16S rRNA gene sequence and their accession numbers published in DNA database.*

Strain ID	Strain / Genus	No. of nucleotides of 16S rRNA gene	Accession No. of 16S rRNA gene	Closely related validly published species	Sequence similarity (%) of 16S rRNA gene
NCCP-132	*Bacillus* sp.	1557	AB562920	*Bacillus pakistanensis* NCCP-168^T (AB618147)	96.66
NCCP-133	*Bacillus* sp.	1477	AB562921	*Bacillus oceanisediminis* H2^T (GQ292772)	99.35
NCCP-134	*Ornithinibacillus* sp.	1538	AB562922	*Ornithinibacillus contaminans* CCUG 53201^T (FN597064)	96.41
NCCP-135	*Oceanobacillus* sp.	1499	AB562923	*Oceanobacillus profundus* CL-MP28^T (DQ386635)	96.75
NCCP-136	*Bacillus* sp.	1436	AB562924	*Bacillus oryzaecorticis* R1^T (KF548480)	98.00

Phylogenetic Analysis of Identified Strains

The strains NCCP-132, NCCP-133 and NCCP-136 belong to genus *Bacillus* based on 16S rRNA gene sequence data as depicted in the phylogenetic tree (*Figure 2*). Strain NCCP-132 showed 96.66 % similarity with *Bacillus pakistanensis* NCCP-168^T (*Table 1*), whereas NCCP-133 and NCCP-136 have sequence similarity of 99.35 % and 98.0 % with *Bacillus oceanisediminis* H2^T and *Bacillus oryzaecorticis* R1^T, respectively. Based on 16S rRNA gene sequence data, the strains NCCP-134 and NCCP-135 belonged to genera *Ornithinibacillus* and *Oceanobacillus* (*Figure 3*) and showed sequence similarity of 96.41 % and 96.75 % with *Ornithinibacillus contaminans* CCUG 53201^T (FN597064) and *Oceanobacillus profundus* CL-MP28^T (DQ386635), respectively. As the similarity with pre-identified species was less than 97 %, suggesting these two strains can be further studied taxonomically to delineate these strains as novel species.

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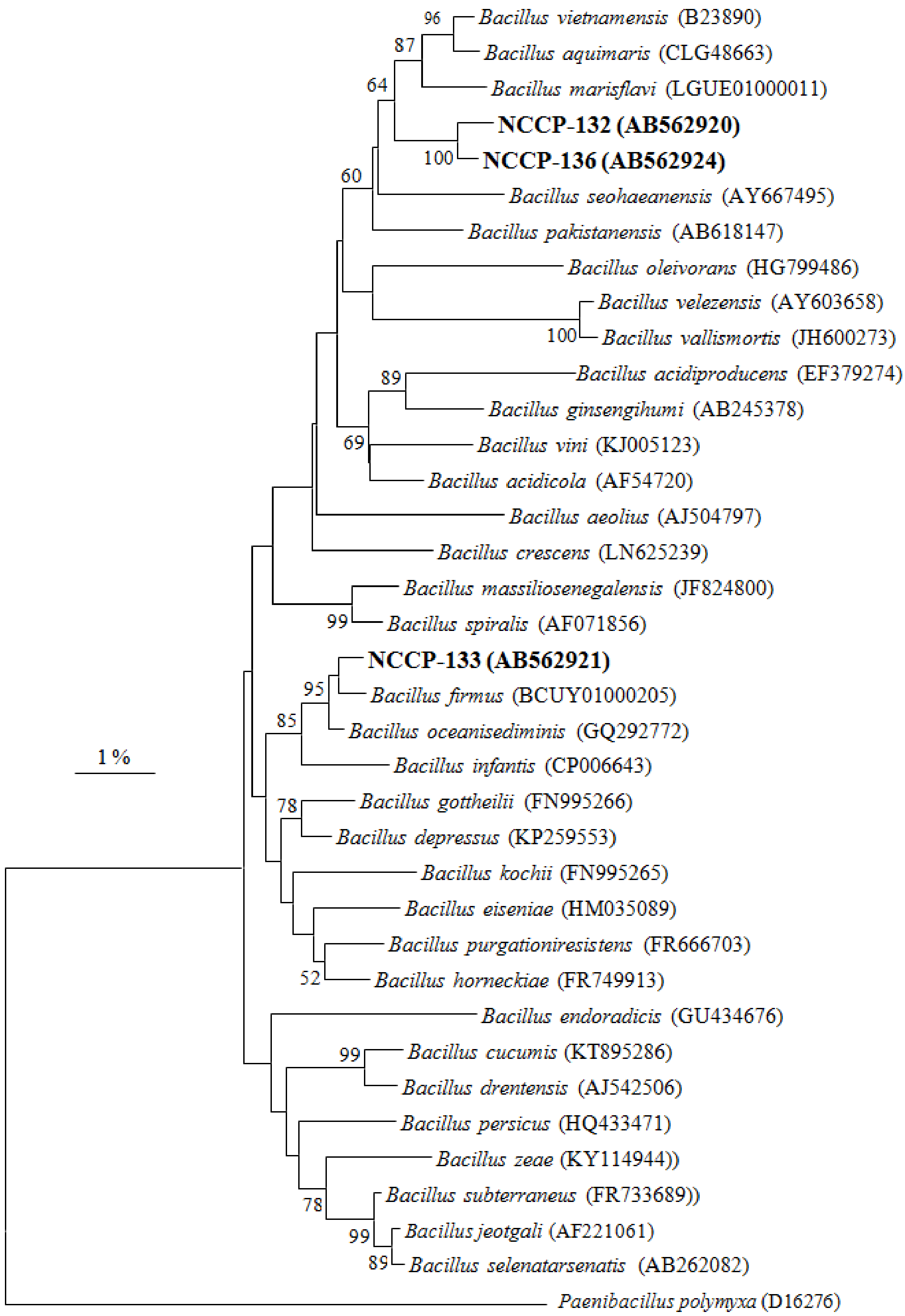

Figure 2. *Phylogenetic tree showing inter-relationship of the strains NCCP-132, NCCP-133 and NCCP-136 with the most closely related Bacillus species inferred from sequences of 16S rRNA gene. Data with gaps were removed during alignment for the construction of tree, which is rooted by using Paenibacillus polymyxa (D16276) as an out group. The tree was generated using MEGA software package based on comparison of approximately 1,240 nucleotides. Bootstrap values (only > 50 % are shown), expressed as a percentage of 1,000 replications, are given at the branching points. The sequence of Bar, 1% sequence divergence. The accession number of each type strain is shown in parentheses.*

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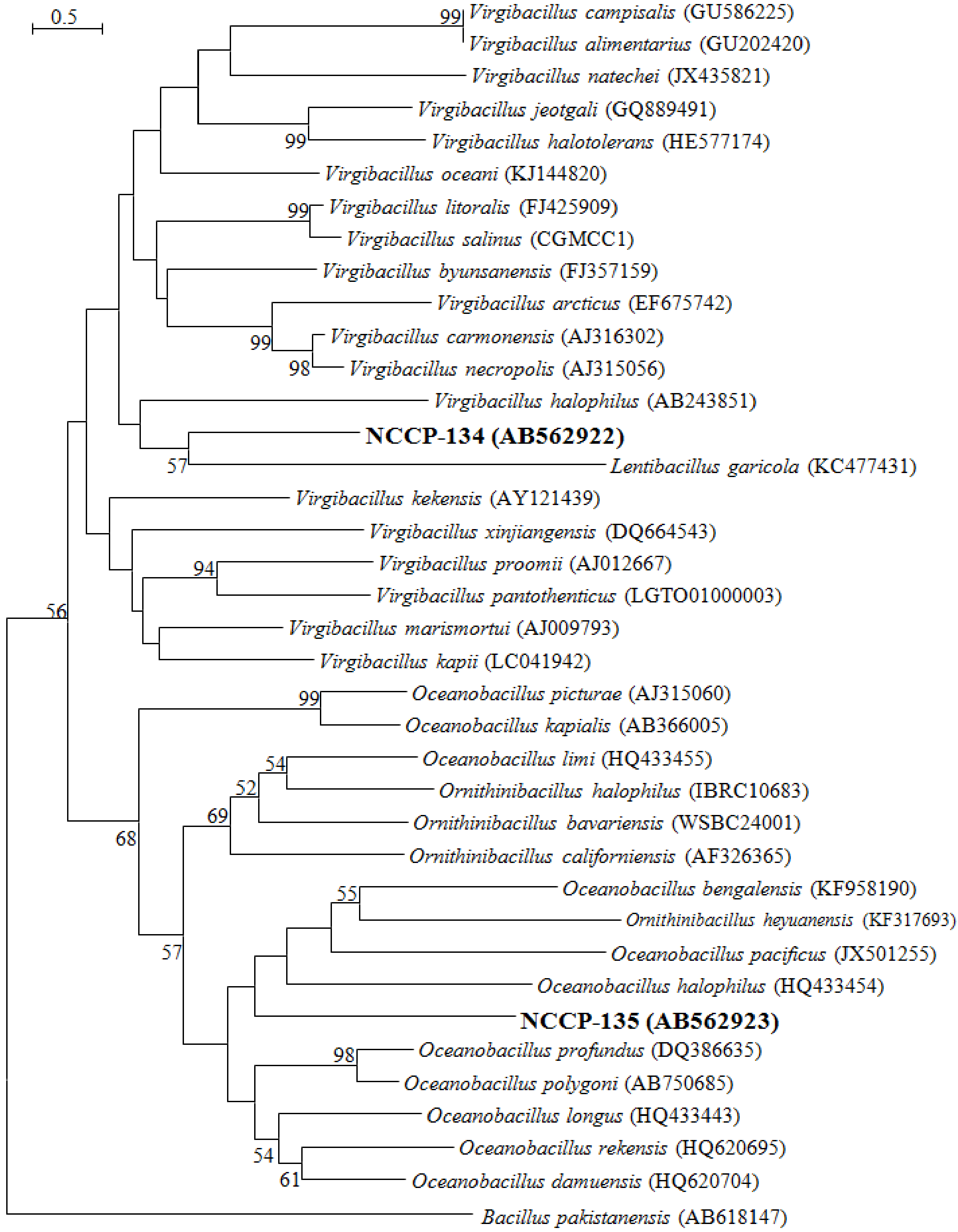

Figure 3. *Phylogenetic tree showing inter-relationship of the strains NCCP-134 and 135 with the most closely related Oceanobacillus, Virgibacillus, and Lentibacillus species inferred from the sequences of 16S rRNA gene. Data with gaps were removed during alignment for the construction of tree, which is rooted by using Bacillus pakistanensis (AB618147) as an out group. The tree was generated using the MEGA software package based on comparison of approximately 1,240 nucleotides. Bootstrap values (only > 50% are shown), expressed as a percentage of 1,000 replications, are given at the branching points. The sequence of Bar, 0.5 % sequence divergence. The accession number of each type strain is shown in parentheses.*

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The G+C content of the DNA of the type strain should be determined by high performance liquid chromatography (HPLC) and is recommended for the description of novel species, and essential for the description of new genera (Logan et al., 2009). As identical or highly similar 16S rRNA gene sequences do not guarantee species identity, DNA-DNA hybridization is essential in case of species description when 16S rRNA gene sequences of the novel strains show < 97 % or less similarity with existing taxa. Several widely used methods do not allow the determination of thermal stability (expressed as ΔTm) of the hybrid, but differences in ΔTm between the hybrid and the homologous duplex are important and can be decisive for taxonomic conclusions. Hence, determination of ΔTm is recommended (Logan et al., 2009).

Nucleic acid fingerprinting is also recommended because DNA fingerprinting methods provide information at the subspecies and strain level. Examples are amplified fragment-length polymorphism (AFLP) analysis, macrorestriction analysis after pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, rep-PCR (repetitive element-primed PCR, directed to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genome), including REP-PCR (repetitive extragenic palindromic-PCR), ERIC-PCR (enterobacterial repetitive intergenic consensus sequences PCR), BOX-PCR (derived from the boxA element) and $(GTG)_5$-PCR, and ribotyping (Logan et al., 2009). DNA-DNA hybridization study, together with DNA fingerprinting and 16S rRNA sequence analysis clearly demonstrate the strains to be novel or already identified and more work has to be done on the phenotypic and genotypic level.

Colony Morphology of Bacterial Strains

The isolated five bacterial strains were grown at 28 °C for 3 days on TSA medium containing 100 mM boron concentration. Phase-contrast microscopy showed colony morphology of isolated strains. Gram staining of bacterial isolates was also performed (*Table 2*). Colonial pigmentation of the isolates included pale yellow, creamy and orange coloration. The colonies were round and punctiform in shape and circular in form. Colony surfaces were smooth and margins were entire. Colonies had raised, flat, and convex elevations. The colonies possessed moist and mucoid texture, whereas the opacity of colonies was found to be translucent and opaque.

Table 2. *Colony morphology of bacterial strains observed 3 days after inoculation and incubation at 28 °C. The medium used was Tryptic Soy Agar (TSA) containing 100 mM boron.*

Characteristics	Bacterial Strains				
	NCCP-132	**NCCP-133**	**NCCP-134**	**NCCP-135**	**NCCP-136**
Color	Cream	Yellow	Cream	Yellow	Orange
Shape	Round	Round	Punctiform	Round	Punctiform
Form	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire	Entire
Surface	Smooth	Smooth	Smooth	Smooth	Smooth
Elevation	Convex	Raised	Flat	Raised	Raised
Texture	Moist	Mucoid	Mucoid	Moist	Moist
Opacity	Translucent	Translucent	Translucent	Translucent	Opaque
Size	2.5 mm	1.5 mm	0.5 mm	1.0 mm	1.0 mm

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Phase-contrast microscopy at x1000 magnification is superior to bright-field microscopy of stained smears, including spore staining, and is much more convenient (Logan et al., 2009). Electron microscopy could be done to reveal additional morphological information like flagellation, and sporangial appearance could also be checked.

Optimization of growth conditions for all strains was carried out regarding pH, temperature and NaCl tolerance (*Table 3*). The boron-tolerant bacterial strains were found to grow best at 7-9 pH, 16-37 °C temperature and 0-4% (v/v) NaCl concentration. All bacterial isolates were oxidase and catalase positive as well as Gram-positive. Positive, negative, weakly positive, moderately positive, and strongly positive observations were recorded via tests of API ZYM and API 50CH kits (*Table 4a, b*).

Table 3. *Physiological characteristics of boron tolerant isolated bacterial strains.*

Bacterial Strains	**Range of growth at Boron concentration (Optimum)**	**Range of growth at NaCl concentration (Optimum)**	**Range of growth at pH (Optimum)**	**Range of growth at Temperature (Optimum)**
NCCP-132	0-450 (100)	0-3 (0-2)	7-8 (7)	10-45 (37)
NCCP-133	0-450 (100)	0-5 (0 -4)	7-9 (8.5-9.0)	10-45 (28)
NCCP-134	0-450 (100)	0-14 (0)	7-8 (8)	10-37 (37)
NCCP-135	0-450 (100)	0-14 (1-2)	5-9 (8)	10-37 (28- 37)
NCCP-136	0-200 (100)	0-7 (0)	7-9 (8.5)	10-45 (28- 37)

Table 4a. *Biochemical results for enzymatic activities of the strains.*

Biochemical Tests	**Bacterial Strains**				
	NCCP-132	**NCCP-133**	**NCCP-134**	**NCCP-135**	**NCCP-136**
Alkaline phosphatase	–	–	++	–	–
Esterase (C4)	–	–	–	–	–
Esterase Lipase (C8)	–	–	w+	w+	–
Lipase (C14)	–	–	–	–	–
Leucine arylamidase	w+	–	–	w+	–
Valine arylamidase	w+	–	–	–	–
Cystine arylamidase	w+	w+	m+	m+	w+
Trypsin	–	–	–	–	–
α-chymotrypsin	w+	w+	m+	m+	w+
Acid phosphatase	–	–	+	–	–
Naphthol-AS-B1-	++	–	–	–	–
α-galactosidase	w+	–	–	–	–
β-galactosidase	w+	–	m+	–	–
β-glucuronidase	–	–	–	–	–
α-glucosidase	w+	–	–	–	–
β-glucosidase	w+	–	–	–	–
N-acetyl-β-glucosaminidase	w+	–	++	w+	–
α-mannosidase	w+	–	–	w+	–
β-galactosidase	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–

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Lysine decarboxylase	–	–	–	–	–
Ornithine decarboxylase	–	–	–	–	–
Citrate utilization	–	–	–	–	–
Urease	–	–	–	–	–
Tryptophane deaminase	+	+	+	+	+
Gelatinase	–	–	–	+	+
α-fucosidase	w+	–	–	–	–

+ positive, - negative, w+ weakly positive, m+ moderately positive, ++ strongly positive

Table 4b. *Biochemical results for utilization of carbohydrates and other substrates.*

Biochemical Tests	Bacterial Strains				
	NCCP-132	**NCCP-133**	**NCCP-134**	**NCCP-135**	**NCCP-136**
Glycerol	–	–	w+	–	–
Erythritol	–	–	–	–	–
D-Arabinose	–	–	–	–	–
L-Arabinose	–	–	w+	–	–
D-Ribose	–	–	w+	–	–
D-Xylose	–	–	–	–	–
L-Xylose	–	–	–	–	–
D-Adonitol	–	–	–	–	–
Methyl-*β*D-Xylopyranoside	–	–	–	–	–
D-Galactose	–	–	–	–	–
D-Glucose	–	–	w+	–	–
D-Fructose	–	–	w+	–	–
D-Mannose	–	–	w+	–	–
L-Sorbose	–	–	–	–	–
L-Rhamnose	–	–	–	–	–
Dulcitol	–	–	–	–	–
Inositol	–	–	–	–	–
D-Mannitol	–	–	w+	–	–
D-Sorbitol	–	–	w+	–	–
Methyl-α-D-Mannopyranoside	–	–	–	–	–
Methyl-αD-Glucopyranoside	–	–	–	–	–
N-Acetyl Glucosamine	–	–	w+	+	–
Amygdalin	–	–	–	–	–
Arbutin	–	–	–	–	–
Esculin	–	–	++	–	–
Salicin	–	–	w+	–	–
D-Cellobiose	–	–	w+	w+	–
D-Maltose	–	–	–	–	–
D-Lactose	–	–	–	–	–

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D-Melibiose	–	–	+	–	–
D-Saccharose	–	–	–	–	–
D-Trehalose	–	–	–	–	–
Innulin	–	–	–	–	–
D-Melezitose	–	–	–	–	–
D-Raffinose	–	–	–	–	–
Amidon(Starch)	–	–	–	w+	–
Glycogen	–	–	–	w+	–
Xylitol	–	–	–	w+	–
Gentiobiose	–	–	–	–	–
D-Turanose	–	–	–	–	–
D-Lyxose	–	–	–	–	–
D-Tagatose	–	–	w+	w+	–
D-Fucose	–	–	–	–	–
L-Fucose	–	–	–	–	–
D-Arabitol	–	–	w+	–	–
L-Arabitol	–	–	w+	–	–
Potassium gluconate	–	–	w+	–	–
Potassium-2-ketogluconate	–	–	–	–	–
Potassium-5-ketogluconate	+	–	–	w+	–
H_2S production	–	–	–	–	–
Indole production	–	–	–	–	–
Acetoin production (Voges Proskauer)	–	–	–	–	–
Fermentation/ oxidation of:					
Glucose	–	–	–	–	–
Mannitol	–	–	–	–	–
Inositol	–	–	–	–	–
Sorbitol	–	–	–	–	–
Rhamnose	–	–	–	–	–
Saccharose	–	–	–	–	–
Melibiose	–	–	–	–	–
Amygdalin	–	–	–	–	–
Arabinose	–	–	–	–	–
NO_2 production	–	–	–	+	+
N_2 production	–	–	–	–	–

+ positive, - negative, w+ weakly positive, ++ strongly positive

Optimization of Conditions for Bacterial Growth and Biochemical Characterization

Chemotaxonomic fingerprinting techniques applied to aerobic endospore-formers include fatty acid methyl ester (FAME) profiling, PAGE analysis of whole-cell proteins, polar lipid analysis, quinone content, cell-wall diamino acid content, pyrolysis mass spectrometry, Fourier-transform infrared spectroscopy, Raman spectroscopy and matrix-

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assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. Fatty acid profiles are very useful in descriptions of new taxa, and it is recommended that the fatty acid profile should be available and the minor compounds that are characteristics of a novel taxon should be stated in the description (Logan et al., 2009).

Description of NCCP-132 and NCCP-136

Phylogenetic analysis revealed that NCCP-132 and NCCP-136 belong to the *Bacillus* genus, and are similar to *B. marisflavi*, *B. aquimaris* and *B. vietnamensis*, based on comparison of 16S rRNA gene sequence. On TSA medium, colonies of NCCP-132 are cream in colour, round, circular, smooth, having entire margins, having convex surface, moist, translucent and 2.5mm in size after 3 days at 28 °C. Cells are gram-positive, catalase-positive and oxidase-positive. In API ZYM gallery, alkaline phosphatase, esterase, esterase lipase, lipase, trypsin, acid phosphatase and β-glucuronidase tests are negative. Leucine arylamidase, valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase tests are weakly positive. Naphthol-AS-B1-phosphohydrolase test is strongly positive. In API 50CH gallery, glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate tests are negative. Potassium-5-ketogluconate test is positive. Optimum boron concentration needed for growth is 0mM and boron range for growth is 0-450 mM. Optimum growth pH is 7.0, thus, neutrophilic. pH range for growth is 7.0-8.0. Optimum growth temperature is 37 °C, thus, mesophilic. The temperature range for growth is 10-45 °C. Optimum NaCl concentration needed for growth is 0-2 %, thus, slightly halotolerant. The growth range for NaCl concentration is 0-3 %.

On TSA medium, colonies of NCCP-136 are orange in colour, punctiform, circular, smooth, having entire margins, having raised surface, moist, opaque and 1.0mm in size after 3 days at 28°C. Cells are gram-positive, catalase-positive and oxidase-positive. In API ZYM gallery, alkaline phosphatase, esterase, esterase lipase, lipase, trypsin, acid phosphatase, leucine arylamidase, α-galactosidase, β-galactosidase, naphthol-AS-B1-phosphohydrolase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase and β-glucuronidase tests are negative. Valine arylamidase, cystine arylamidase, α-chymotrypsin tests are weakly positive. In API 50CH gallery, glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate tests are negative. Optimum boron concentration needed for growth is 0mM and boron range for growth is 0-200mM. Optimum growth pH is 8.5, thus, alkaliphilic. pH range for growth is 7.0-9.0. Optimum

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growth temperature is 28-37°C, thus, mesophilic. The temperature range for growth is 10-45°C. Optimum NaCl concentration needed for growth is 0%, thus, non-halotolerant. The growth range for NaCl concentration is 0-7%.

Description of NCCP-133

Phylogenetic analysis revealed that NCCP-133 belongs to the *Bacillus* genus, and is similar to *B. firmus*, based on comparison of 16S rRNA gene sequence. On TSA medium, colonies of NCCP-133 are yellow in colour, round, circular, smooth, having entire margins, having raised surface, mucoid, translucent and 1.5mm in size after 3 days at 28°C. Cells are gram-positive, catalase-positive and oxidase-positive. In API ZYM gallery, alkaline phosphatase, esterase, esterase lipase, lipase, trypsin, acid phosphatase, leucine arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, naphthol-AS-B1-phosphohydrolase, α-mannosidase, α-fucosidase, N-acetyl-β-glucosaminidase, α-glucosidase, β-glucosidase and β-glucuronidase tests are negative. Cystine arylamidase and α-chymotrypsin tests are weakly positive. In API 50CH gallery, glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium-2-ketogluconate and potassium-5-ketogluconate tests are negative. Boron range for growth is 0-450mM. Optimum pH is 8.5-9.0, thus, alkaliphilic. pH range for growth is 7.0-9.0. Optimum temperature is 16-32°C, thus, psychrophilic and mesophilic. The temperature range for growth is 10-45°C. Optimum NaCl concentration is 0-4%, thus moderately halotolerant. The growth range for NaCl concentration is 0-5%.

Description of NCCP-134

Phylogenetic analysis revealed that NCCP-134 belongs to *Lentibacillus* genus, and is similar to *L. garicola*, based on comparison of 16S rRNA gene sequence. On TSA medium, colonies of NCCP-134 are cream in colour, punctiform, circular, smooth, having entire margins, having flat surface, mucoid, translucent and 0.5mm in size after 3 days at 28°C. Cells are gram-positive, catalase-positive and oxidase-positive. In API ZYM gallery, esterase, lipase, trypsin, leucine arylamidase, valine arylamidase, naphthol-AS-B1-phosphohydrolase, α-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase and β-glucuronidase tests are negative. Alkaline phosphatase and N-acetyl-β- glucosaminidase tests are strongly positive. Esterase lipase test is weakly positive. Cystine arylamidase, α-chymotrypsin and β-galactosidase tests are moderately positive. In API 50CH gallery, erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, arbutin, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, amidon, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, potassium-2-ketogluconate tests are negative. Glycerol, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, N-acetylglucosamine, salicin, D-cellobiose, D-tagatose, D-arabitol, L-arabitol and potassium gluconate tests are weakly positive. D-melibiose test is positive. Esculin test is strongly positive. Optimum

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boron concentration needed for growth is 0mM and boron range for growth is 0-450mM. Optimum pH is 8.0, thus, alkaliphilic. pH range for growth is 7.0-8.0. Optimum temperature is 37°C, thus, mesophilic. The temperature range for growth is 10-37°C. Optimum NaCl concentration is 0%, thus, non-halotolerant. The growth range for NaCl concentration is 0-14%.

Description of NCCP-135

Phylogenetic analysis revealed that NCCP-135 belongs to the *Oceanobacillus* genus, and is similar to *O. profundus*, based on comparison of 16S rRNA gene sequence. On TSA medium, colonies of NCCP-135 are yellow in colour, round, circular, smooth, having entire margins, having raised surface, moist, translucent and 1.0mm in size after 3 days at 28°C. Cells are gram-positive, catalase-positive and oxidase-positive. In API ZYM gallery, alkaline phosphatase, esterase, lipase, acid phosphatase, trypsin, valine arylamidase, naphthol-AS-B1-phosphohydrolase, α-galactosidase, α-glucosidase, β-glucosidase, α-fucosidase and β-glucuronidase, β-galactosidase tests are negative. Esterase lipase and leucine arylamidase, α-mannosidase, N-acetyl-β-glucosaminidase tests are weakly positive. Cystine arylamidase, α-chymotrypsin and tests are moderately positive. In API 50CH gallery, glycerol, L-arabinose, D-ribose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, esculin, salicin, D-melibiose, erythritol, D-arabitol, L-arabitol, potassium gluconate, D-arabinose, D-xylose, D-adonitol, methyl-βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, amygdalin, arbutin, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, L-xylose potassium-2-ketogluconate tests are negative. Potassium-5-ketogluconate, D-cellobiose, D-tagatose, amidon, glycogen, xylitol and tests are weakly positive. N-acetylglucosamine test is positive. Optimum boron concentration needed for growth is 0-50 mM and boron range for growth is 0-450mM. Optimum pH is 8.0, thus, alkaliphilic. pH range for growth is 5.0-9.0. Optimum temperature is 37 °C, thus, mesophilic. The temperature range for growth is 10-37°C. Optimum NaCl concentration is 1-2%, but its tolerance to NaCl ranges 0-14%, thus, slightly halotolerant.

Conclusion

Bacterial isolates having high biological diversity were found from the sewage samples taken from different sites of sewage treatment pond. Biological diversity is evident from the data depicting morphology of these isolates. As phenotypic characterization was not enough for microbial identification so different factors like pH, temperature and NaCl were optimized for growing boron-tolerant bacteria. Furthermore, biochemical testing showed different biochemical properties of bacterial isolates. However, the accurate taxonomic position of bacterial strains was confirmed by the conventional method for microbial identification, i.e., phylogenetic analysis using comparative sequence analysis of 16S rRNA gene. Out of the five bacterial isolates, four were highly boron-tolerant and one was moderately boron-tolerant strain. NCCP-133 and NCCP-136 were found to be pre-identified strains as these shared > 97 % similarity of 16S rRNA gene sequence with their closest relatives. NCCP-132 (*Bacillus* sp.), NCCP-134 (*Lentibacillus* sp.) and NCCP-135 (*Oceanobacillus* sp.) were found to be novel species based on phylogenetic analysis, however further taxonomic

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characterization particularly chemotaxonomic profiling is required to meet the minimum standards for delineation of these isolates as a novel species.

The present study is the first study from Pakistan exploring a new aspect of extremophiles. Finding out the boron tolerance and essentiality level for novel bacterial strains would provide a genetic resource to identify the genes responsible for mechanisms of boron-tolerance and boron-requirement in bacteria. Gene identification would help in the successful management of boron in agriculture and such genes might be useful for cloning in other organisms especially the crop species that are grown on high boron soils. Moreover, it will provide information to study the biochemistry of boron in living cells.

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SEASONAL PATTERNS IN THE DIVERSITY OF HISTERID BEETLES (HISTERIDAE) ARE ECOSYSTEM SPECIFIC? A CASE IN PARA STATE, NORTHERN BRAZIL

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Abstract. The objective of this work was to characterize the seasonal variation in the community of Histerid beetles (Histeridae) of different ecosystems in the Brazilian Amazon, ranging from primary or secondary forests with different stages of regeneration to cocoa farms and pastures. Pitfall traps were distributed within the monitored ecosystems during different periods of the year. A total of 1945 Histerid beetles, belonging to five genera and 14 species were captured. Higher diversities were observed during the rainy season, although with differences among ecosystems. The genera *Phelister* and *Hister* were ubiquitous in all ecosystems, constituting 71% of all the specimens captured. Histerid beetle communities, discriminated by ordination methods, change gradually from the most conserved ecosystems to more disturbed ones. Moreover, the results showed that disturbed ecosystems, namely cocoa farms and pastures, have detrimental effects on the occurrence of Histerid beetles, which are considered forest specialists, while enhancing generalist species.
Keywords: *Amazon rainforest, biodiversity, soil insect, fluctuation, tropical forest*

Introduction

Currently, there is enormous concern and speculation about the effects of anthropogenic disturbance on the biodiversity of tropical ecosystems, including the effects on species composition and the modifications in the ecological services provided (e.g. Morris, 2010; Aerts and Honnay, 2011; Cajaiba et al., 2017a). Deforestation in the Neotropics, particularly in the Brazilian Amazon (Amazonia), has been causing a sharp erosion of biodiversity and disruption of the complex global climate phenomena (e.g. Hassan et al., 2005; Morris, 2010). Therefore, the conservation of the Amazonian ecosystems represents a growing core challenge for sustaining earth´s functioning and ultimately that of mankind (e.g. Viana and Pinheiro, 1988; Viegas et al., 2014).

Predicting the ecological consequences of land use/land cover (LU/LC) changes is therefore a subject of scientific and political interest in order to support strategic options for sustainable development, land use planning and natural resources management

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(Turner et al., 2007). In this context, ecological assessment and monitoring are important tools to support effective management of ecosystems and natural resources, in which the use of pertinent indicators is crucial for measuring and evaluating the status and trends of target environmental systems (Cajaiba and Silva, 2017).

Ecological integrity is a key concept in natural resource management, and researchers have been trying to improve public understanding of this concept by using simple scientific measures known as ecological indicators (Andreasen et al., 2001). In fact, ecological integrity can be measured and interpreted by changes in abundance, diversity, and composition of groups of indicator organisms that ultimately depend on system resources and conditions. Correlates of integrity in terrestrial landscapes have been proposed, along with many specific indicators that measure various aspects of the ecosystems (Cajaiba and Silva, 2017). Among the various indicators, terrestrial invertebrates, and particularly insects, play a crucial role in most ecological processes, and they are key components of ecosystem structure and functioning (Bicknell et al., 2014; Viegas et al., 2014; Cajaiba and Silva, 2015; Cajaiba et al., 2015; Campos and Hernández, 2015). Insect abundance and richness are related with other taxa, climate, and soil characteristics, thus representing potential target indicators of environmental changes (e.g. Nichols et al., 2008; Cajaiba et al., 2015). Therefore, a better understanding of the ecological relevance of insects in the humid tropics could support decision-making and robust management/recovery of imperilled ecosystems in the scope of the need for rapid, standardised, and cost-effective assessment methodologies (Godfray et al., 1999).

Beetles have characteristics that make them appropriate for ecological studies (Vasquez-Velez et al., 2010; Paoletti et al., 2010; Cajaiba and Silva, 2015) monitoring different compartments of the system (Marinoni, 2001). Among beetles, those of the family Histeridae (Histerid beetles - HB) are known as generalist predators, with wide range of habitats, which may occur in faeces, fungi, tree trunks, decomposing fruit, roots of trees, bird nests, burrows of mammals or reptiles, and in decaying vegetation. In addition, they are important predators of eggs and larvae, particularly of Diptera (Cyclorrhapha). Some groups are related to other animals, particularly social insects such ants and termites (Leivas et al., 2013). Despite its ecological importance, few studies have been conducted in Brazil using this group, and the published ones focused on the systematics (Degallier et al., 2011; Corrêa et al., 2012; Leivas et al., 2012a, b; Leivas et al., 2015).

In the present study, we aimed to characterize the seasonal communities of HB of different ecosystems in the Brazilian Amazon, ranging from primary or secondary forests with different stages of regeneration, to cocoa farms and pastures. These ecosystems, characterized by dissimilar structure and complexity, could be associated with divergent seasonal patterns in richness and diversity. Specifically, we addressed the following questions: 1) does the composition of Histerid beetle communities vary among the different ecosystems and 2) does the diversity of Histeridae assemblies in the Amazon biome differ among seasons?

Material and methods

Study area

This study was performed near the city of Uruará, state of Pará, northern Brazil (*Fig. 1*). The territorial extension of the municipality is 10796 km² and its population encompasses circa 44789 inhabitants. The dominant land use/ land cover (LU/LC) is natural forest (69% of the area) and deforestation is concentrated mainly in the south-central part of territory and near the main roads. Extensive livestock production and the exploitation of timber at a large scale (mostly illegal) are currently considered the most serious environmental threats (Cajaiba and Silva, 2017). The studied areas contain the pertinent gradients, in terms of biophysical and ecological characteristics, for testing the response of Histerid beetle communities (Cajaiba and Silva, 2017) facing the main anthropogenic drivers. These gradients encompass: Native Vegetation - NV, Early Secondary succession – ES (vegetation with five years of regeneration), Mature Secondary succession - MS (vegetation with 15 years of regeneration), Agriculture - Ag (cocoa plantations, Theobroma cacao L.) and Pasture for extensive livestock - Pa. The climate of the study area is classified as Aw (Köppen), hot and humid and the average annual rainfall is 2000 mm (Cajaiba et al., 2017b).

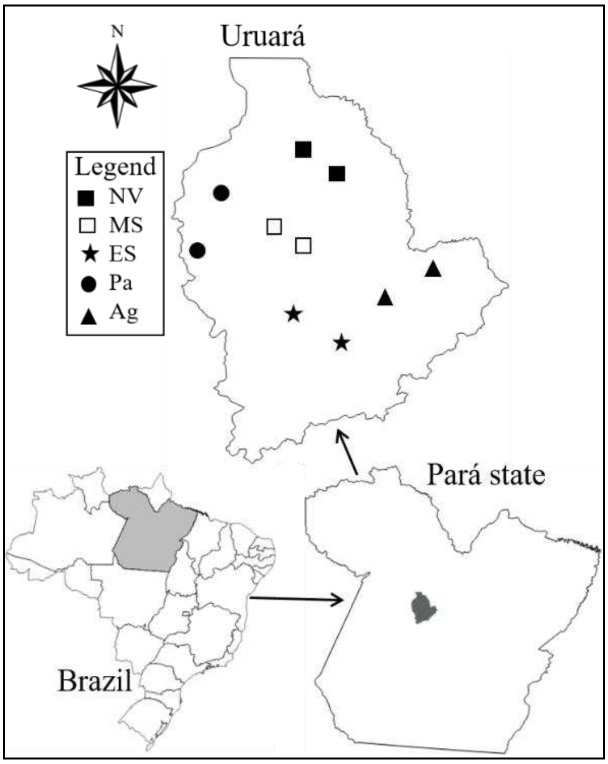

Figure 1. Location of the study region in the municipality of Uruará, state of Pará, northern Brazil. Location of the sampling areas: NV, Native vegetation; MS, Mature secondary succession; ES, Early secondary succession; Ag, Agriculture; Pa, Pasture.

Histerid beetles sampling

Sampling was carried out during the year 2015, in the months of February/ March (rainy season), June (final of rainy season and early dry season) and September/ October (dry season). This allowed integrating eventual seasonal differences in the diversity of Histerid beetles (HB). The sample points were placed at a minimum distance of 100 meters from ecotones, to ensure that most HB captured were associated to the ecosystem in study. Pitfall traps with 75 mm diameter and 110 mm deep were filled with preservative liquid consisting of formalin, alcohol, water and a few drops of

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detergent to break the surface tension. A roof was attached to each trap to prevent rainwater from entering the trap, remaining installed for 48 h prior to collection. Each pitfall contained different baits such as human faeces, meat and banana in order to attract different species according to their feeding habits (non-baited pitfalls were used as control).

In each studied ecosystem (NV, MS, ES, Ag and Pa) seven sample points were placed 100 m apart. Each sample point contained four pitfall traps including the different baits (faeces, carrion, banana and non-baited), separated by 5 meters. The distance between pitfall traps allowed individuals to choose their preferential food resource (Almeida and Louzada, 2009). This protocol was applied to all areas and periods of collections, totalizing a sampling effort of 840 traps. In order to complement the pitfall derived information (Cajaiba et al., 2014), specific methods were used to collect leaf litter invertebrates. Ten random sampling points of 1 m^2 were selected in each ecosystem and sampling period. At each collection point ('litter-only'), only the loose soil was gently scraped with a metal trowel, to include those HB into the samples that fell out of the leaf litter during this collection procedure. We refrained from simply separating leaf litter and soil, considering that when we remove the litter, HB might flee the litter and hide in the loose topsoil.

Assemblage analysis

Richness and abundance of HB were measured in each sampling site and differences among ecosystems were gauged using One-Way-Analysis-of-Variance (ANOVA). ANOVA was also applied to find possible differences in HB along the year. When the ANOVA indicated differences in the average among ecosystems and/or seasonality, a Tukey's post-hoc test was performed to find specific difference(s). The normality of the data was verified by the Shapiro-Wilk test.

The taxonomic composition of HB communities between ecosystems was compared using Permutational Multivariate Analysis of Variance (PERMANOVA). Non-metric Multidimensional Scaling (NMDS) plots were used to help interpret the results found with the PERMANOVAs (see Anderson, 2001 for similar procedure). In order to check for environmental variables with influence on the HB communities, associated with periods of the year, a correlation analysis (Pearson correlation) was applied between HB abundance and richness and meteorological data - temperature, humidity and precipitation. All analyses were performed using PAST software version 3.14 (Hammer et al., 2001).

Results

Composition of Histeridae

A total of 1945 individuals Histerid beetles (HB) were captured, distributed by five genera and 14 species/ morphospecies (species). All species (14 species, 682 individuals) were monitored in the native vegetation (NV), 12 species (513 individuals) in the Mature Secondary succession (MS), 13 species (180 individuals) in Early Secondary succession (ES), 11 species (232 individuals) in Agriculture (Ag) and 13 species (338 individuals) in Pasture (Pa). The most abundant species were *Phelister* sp1 (302 individuals), *Phelister* sp4 (257 individuals), *Hister* sp1 (246 individuals) and *Phelister* sp2 (184 individuals). These four species represent more than 50 percent of all specimens collected. The genera *Phelister* and *Hister* were prevalent in all communities, with more 71 percent of all specimens captured (*Table 1*).

Table 1. *Total numbers of Histerid beetles species in the environments different in the Brazilian Amazon. NV = Native Vegetation; MS = Mature Secondary succession (15 years of regeneration); ES = Early Secondary succession (5 years of regeneration); Ag = Agriculture (Cocoa); Pa = Pasture. R = Rainy Season; Intermediary Season; D = Dry Season.*

Species	**NV**			**MS**			**ES**			**Ag**			**Pa**			**Total**
	R	**I**	**D**	**R**	**I**	**D**	**R**	**I**	**D**	**R**	**I**	**D**	**R**	**I**	**D**	
Phelister haemorrhous	14	4	6	-	-	-	-	-	-	-	-	-	6	4	3	37
Phelister sp1	60	68	-	16	80	11	17	6	3	-	6	8	7	20	-	302
Phelister sp2	26	25	-	26	20	-	9	-	2	3	2	34	7	15	15	184
Phelister sp3	-	25	-	10	72	22	1	2	1	-	6	10	10	5	6	170
Phelister sp4	28	20	6	35	30	-	2	10	2	-	-	26	19	20	59	257
Hister punctifer	24	8	7	14	10	3	6	6	7	6	6	5	3	2	1	108
Hister sp1	44	30	-	33	38	18	18	6	-	9	14	30	-	2	3	246
Hister sp2	6	40	-	2	2	1	-	-	-	8	1	8	2	-	18	88
Omalodes marseuli	11	8	7	13	2	6	8	2	1	7	3	3	10	4	4	89
Omalodes sp1	72	14	6	2	-	-	4	8	4	4	4	10	6	2	12	148
Omalodes sp2	30	24	16	10	10	0	1	-	8	-	-	-	3	-	24	126
Euspilotus sp1	16	8	8	5	2	1	6	6	5	6	6	2	5	4	-	80
Euspilotus sp2	6	6	4	10	5	4	12	11	7	6	3	1	14	7	7	103
Operclipygus sp	5	-	-	-	-	-	2	-	-	-	-	-	-	-	-	7

Histerid beetles (HB) richness and abundance presented significant differences between ecosystems ($F_{4,114} = 111.3$, $p<0.001$; $F_{4,114} = 346$, $p<0.001$) (*Figs. 2a and 2b*). Generally, less disturbed ecosystems had higher values of both indexes, although with exceptions: higher richness and abundance depicted in Pastures when comparing with Cocoa farms (Ag) and Early secondary succession (ES).

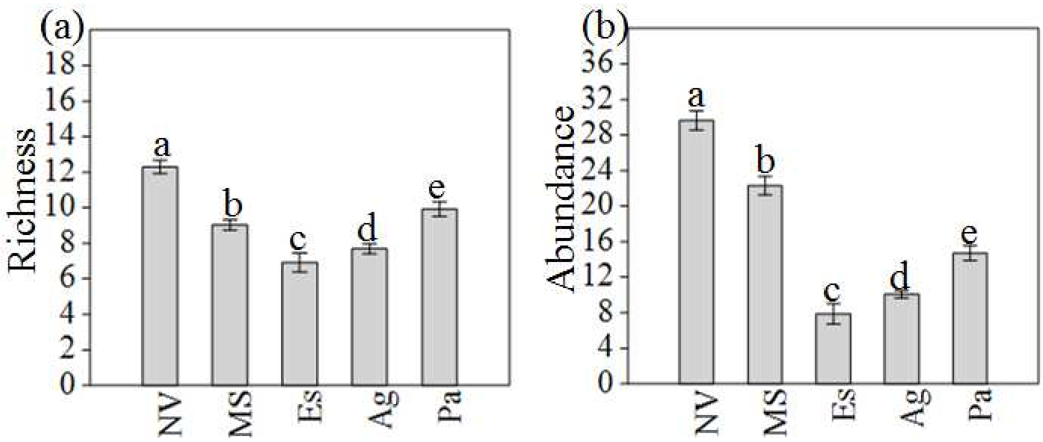

Figure 2. *Differences in the projected values for (a) Richness (± SE), and (b) Abundance (± SE) of Histerid beetles (HB) community in the different ecosystems considered. The values followed by the same letters are not significantly different according to Tukey test. NV, Vegetation native; MS, Mature Secondary succession (vegetation with 15 years of regeneration); ES, Early Secondary succession (vegetation with five years of regeneration); Ag, Agriculture; Pa, Pasture.*

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NV presented all species while *Phelister haemorrhous* was not captured in MS, ES and Ag; *Operclipygus* sp was not captured in MS, Ag and Pa; *Hister* sp2 and *Omalodes* sp2, were not found in ES and Ag, respectively.

The results of the NMDS showed that the HB assemblages of different ecosystems could be separated from each other by ordination of the species composition, suggesting that these assemblages change gradually from the most pristine to more disturbed ecosystems. The composition of the HB of NV and MS were more similar to each other than HB assemblages of other habitats: ES, Ag and Pa could be discriminated from NV and MS (*Fig. 3*). In fact, the Permutational Multivariate Analysis of Variance (PERMANOVA) showed that HB taxonomic composition of the ecosystems studied was significantly different ($F_{4,114} = 28.44$, $p<0.0001$).

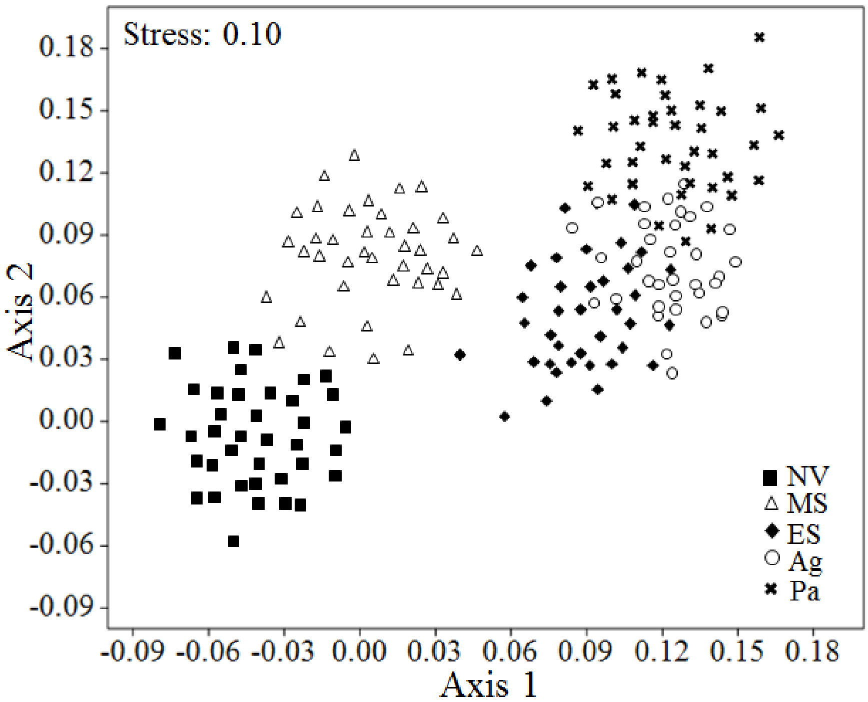

Figure 3. *Non-metric multidimensional scaling (NMDS) showing Histerid beetles grouped in accordance with the ecosystems (using Bray-Curtis similarity). NV, Vegetation native; MS, Mature Secondary succession (vegetation with 15 years of regeneration); ES, Early Secondary succession (vegetation with five years of regeneration); Ag, Agriculture (Cocoa); Pa, Pasture.*

Seasonality patterns in Histerid beetles assemblages

With respect to the relation between the period of the year and HB assemblages, statistical differences in abundance and richness were identified ($F_{2,342} = 11.71$, $p<0.001$; $F_{2,342} = 13.29$, $p<0.001$): significant differences in abundance were found between the averages of the rainy and dry seasons (Tuckey test: Q=6.76, p<0.001), and the intermediary and dry seasons (Tuckey test: Q=5.64, p<0.05); significant differences in richness were found between averages of rainy and intermediary seasons (Tuckey test: Q=3.17, p<0.01), and intermediary and dry seasons (Tuckey test: Q=4.09, p<0.05) (*Fig. 4a*).

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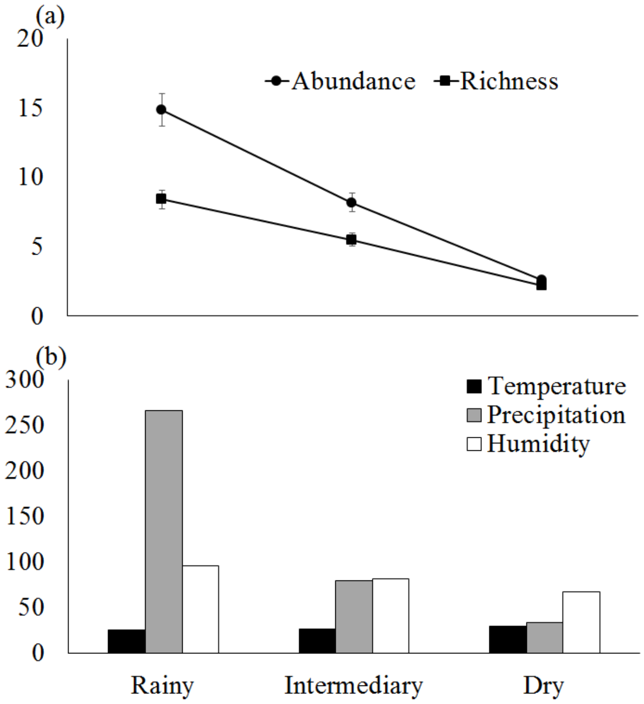

Figure 4. *Abundance and richness of Histerid beetles (a) and distribution of environmental variables during the study period (b).*

Among the climatic variables evaluated (*Figure 4b*), humidity and precipitation were positively correlated with the abundance and richness of Histeridae. In contrast, the air temperature negatively influenced these indexes (*Table 2*).

Table 2. *Pearson correlation between environmental variables and ecological indices (abundance and richness) of Histeridae collected in Uruará, Pará, northing Brazil. Asterisks indicate statistical significance at level of * p<0.05; ** p<0.01;*** p<0.001.*

Environmental variables	Abundance	Richness
Humidity	0.57***	0.49***
Temperature	-0.29*	-0.29**
Precipitation	0.48***	0.44***

Discussion

Composition

Degallier et al. (2011) highlighted the shortage of information on Histerid beetles (HB) in Neotropical areas, where environmental and ecological conditions should enhance their biodiversity. In fact, there is a lack of specific collections and specialized taxonomists for this group. Despite the lack of HB collections, the results obtained provide clues on the diversity of the group in the Amazon region (Leivas et al., 2013).

The genus *Phelister* (Marseul, 1853), which was the most abundant in the present study, represents a varied genus with 88 species described with mostly Neotropical distribution (Mazur, 2011). These species can be found on diverse substrates like faeces, carcasses, decomposing plants, plants, mammal burrows, bird nests, and in debris piles

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of leaf-cutting ants. Some species, associated with carcasses, have been considered relevant for forensic entomological studies (Almeida and Mise, 2009; Leivas et al., 2013). The enormous diversity, lack of taxonomic studies, and the possibility of description of many new taxa hinders the identification of species (Leivas et al., 2013). The genus *Hister* (Linnaeus, 1758), which was the second most abundant in the present study, is the most diverse genus of HB, with approximately 195 described species distributed across all zoogeographic regions (Mazur, 2011). These species occur in faeces, carcasses, decomposing plants and fungi, mammal burrows, and in debris piles of leaf-cutting ants. The small number of species recorded in Brazil suggests that new taxa and new records might be described in future (Leivas et al., 2013, 2015).

In the present study, both richness and abundance were the highest in the most pristine and/or conserved ecosystems (e.g. NV and MS). These results suggest the presence of differences among HB communities associated with specific ecosystems, probably related with the environmental conditions and bioecology of species (Moraes et al., 2013). Summerlin (1989) mentions a trend starting from low dominance and high richness of HB communities in pristine ecosystems to higher abundances and reduced richness of HB communities in open and degraded ones. Thus, diversity and abundance of HB communities are intimately related with differences in environmental characteristics of these ecosystems (Lopes et al., 2005).

Biotic and abiotic factors associated with most pristine/ heterogeneous environments, are, for example, mild climates, high canopy cover, and complex ground cover that provide environments for hibernation and more diverse prey, and better conditions for larvae and adults. Cattle treading on pasture areas or the use of machinery in agriculture areas contribute to soil compaction, resulting in changes in the soil structure, reduction of soil litter and water, thereby decreasing and/or eliminating many species (Kruess and Tscharntke, 2002).

Seasonality

In the present study, the abundance and richness of HB collected at different times of the year (rainy season, intermediary season, and dry season) depicted a trend with higher diversity during the rainy season, corroborating the hypothesis that the abundance, richness, and diversity are associated with climatic conditions (Andresen, 2005). Nonetheless, microclimatic factors are not necessarily correlated with climatic conditions, but they play an important part in the activity of HB and associated taxa in different climates (Moraes et al., 2013).

The positive correlation between air moisture and the diversity of HB is expected, because higher air moisture may produce favourable microclimates for HB. Previous studies demonstrated that vegetation structure and its effects on microclimate (e.g. temperature and air moisture) might be one of the most important factors controlling and structuring the distribution of beetles (Magura et al., 2000). Apparently, the evolutionary life cycle strategy of beetles is optimized and synchronized with seasonal changes of microclimatic environmental conditions (Kotze et al., 2011; Wang et al., 2014).

Although temperature was negatively correlated with abundance and richness in the present study, annual temperature variation in this region is small, suggesting that rain and humidity are probably the main drivers affecting the dynamics of invertebrates (Andresen, 2008). Silva et al. (2010) presented two hypotheses to explain the lower abundance and richness of adult beetles in the dry season: (1) adults are sensitive to the

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effects of drought and remain underground during this period, or (2) adults die in the dry season and only the immature beetles survive in the nest, reaching the adult stage at the beginning of the wet season. Nevertheless, temperature is a major factor affecting activity, flight, foraging behaviour, and metabolism of beetles (Saska et al., 2010).

Additional studies are necessary to document the extent of sensitivity to microclimate and climate, and they could be particularly relevant in the light of the potential effects of climate change (Williams et al., 2007; Maveety et al., 2014). Understanding seasonal patterns of beetles in a given region is important for several reasons. Firstly, seasonal variations in abundance, richness, and species composition emphasize the role of phenology and the effect of survey timing on the results obtained for studying the association of HB with ecosystems. Further, seasonal information of HB might be a relevant ecological indicator, which might have importance in the management of ecosystems.

Conclusion

The Histerid beetles collected in this study demonstrate patterns of habitat preference and marked seasonal variations. In addition, the results indicate factors and conditions that might affect overall biodiversity distribution in the Brazilian Amazon. In fact, the present study should be complemented with studies linking Histerid beetles with other taxa for understanding and assessing the state of conservation of the diverse ecosystems of the studied region.

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PREDICTION OF NO_2 CONCENTRATIONS IN A GAS REFINERY USING AIR DISPERSION MODELING

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Abstract. Nitrogen dioxide (NO_2) is considered as one of the most important ambient air pollutants in gas refineries. In this study, AERMOD dispersion model was applied for prediction of NO_2 ambient concentrations and dispersion patterns from point sources, including 13 stacks and six flares in a gas refinery located in Asaluyeh, Iran. For this purpose, the NO_2 concentrations exhausted from the stacks were measured by a portable emission analyzer and the NO_2 concentrations resulted from the flares were estimated using the emission factors. Moreover, the amounts of ambient NO_2 concentrations in 10 monitoring stations were measured in four seasons. Then, the ambient NO_2 concentrations and dispersion patterns were predicted using the AERMOD model within a domain of 10 × 10 km^2, in 1-hr and 12 months averages and the unhealthy zones in the study area were defined. The results revealed that for both annual observed and predicted values, ambient NO_2 concentrations were higher than WHO standard limits but they did not exceed the US EPA standard limits. However, the hourly observed and predicted concentrations were lower than the standard levels. Statistical methods were used for comparing the predicted and observed NO_2 concentrations. Simulation results indicated that the predicted concentrations were underestimated by a factor of 20 % in comparison to the measured ones which revealed the estimated contribution of other sources including mobile sources and neighbor sources located in the vicinity of the gas refinery.
Keywords: *AERMOD, air quality, emission, performance, point source*

Introduction

Air pollution will adversely affect the quality of life and human health. Therefore, it has been widely concerned by environmental experts in the world.

The World Health Organization (WHO) has reported that about 2.7 million people die due to the health effects of air pollution annually (WHO, 2014). The minimum concentrations of gasses in the atmosphere could be essential in determining the health status of the societies (Shooter et al., 1993). Meanwhile, the air quality management policies are important in order to reduce the acute effects of air pollutants. Identifying the emissions due to different sources and assessing their adverse effects are important for appropriate air quality management (Bhanarkar et al., 2005).

The NO_x concentrations exhausted from the gas refinery usually increase with rising ignition temperature that leads to producing NO, and then oxidized to nitrogen dioxide (NO_2) in the presence of oxygen in the atmosphere. NO_2 is an air pollutant with a 1–3 days

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lifetime in mesoscale. Ambient NO_2 concentrations have been regulated by the U.S. EPA as one of the six primary air pollutants. Respiratory tract infections due to the pollutant's interaction with the immune system may be increased by NO_2 exposure. Furthermore, it has been associated with slight respiratory symptoms at low NO_2 concentrations and with death in indoor locations (Chen et. al, 2007). It is caused throat, eyes and nose irritation for inhalation in human (Perkins, 1974). Direct effects of nitrogen oxides can be considered as the creation of smog and photochemical ozone components (Akdemir et al., 2013). The U.S. EPA (2010) has determined that the hourly average ambient NO_2 concentrations should not exceed 200 $\mu g/m^3$. In order to meet the regulation goals and for minimizing the adverse effects of air pollutants, it is essential to study the dispersions of air pollutants which are affected by several factors including atmospheric stability, orography, obstacles, roughness, maximum mixing height, wind speed and wind direction, and height of the release above ground level (Crowl and Louvar, 2002; Seangkiatiyuth et al., 2011).

Various methods including measurement, emission inventory and simulation studies are applied to assess air pollution impacts due to pollutants released into the atmosphere and to monitor whether pollutants' concentrations exceed the standard limits (Capelli et al., 2013). Air quality dispersion modeling is important for predicting the pollutants' concentrations, simulating the emission distribution patterns and the spatial allocation of outdoor air pollutants (Holmes and Morawska, 2006; Zhang et al., 2010). On the other hand, dispersion models are effective tools for predicting the contributions of emissions due to various pollutant sources including traffic, industry, residential and commercial sectors (Cimorelli et al., 2005). Advanced dispersion models such as American Meteorological Society/Environmental Protection Agency Regulatory Model (AERMOD) were often applied for predicting the air quality after a long time, e.g. at least 10 years (Ma et al., 2013).

As AERMOD model can combine various concepts and complex algorithms, it has been used for evaluating the ambient concentrations and dispersion patterns of some pollutants, including SO_2, PM_{10}, VOCs, hydrogen cyanide (HCN),and sulfur hexafluoride (SF_6) (Bhardwaj et al., 2005; Orloff et al., 2006; Venkatram et al., 2001, 2004, 2009; Zou et al., 2009, 2010).

Moreover, AERMOD can be used for simulating the dispersion of heavy metals, such as chromium and mercury (Sax and Isakov, 2003; Mazur et al., 2009). When upper climatological data are not accessible, AERMOD can be integrated with meteorological models such as MM5, WRF, and RAMS, etc (Caputo et al., 2003; Isakov et al., 2007; Kesarkar, 2007).

Some studies were carried out regarding prediction of NO_2 concentrations and dispersion patterns with AERMOD. The dispersion of NO_2 emitted from four cement plants in Thailand were studied using AERMOD model. In this study, the predicted ambient NO_2 concentrations were compared with the observed values in 12 receptors. The results showed that simulated and observed concentrations were in better agreement within 1-5 km than those at receptors situated in 5km further away from the reference point. Moreover, the results revealed that both observed predicted ambient NO_2 concentrations were not exceed the limit values set by the National Ambient Air Quality Standards (Seangkiatiyuth et al., 2011). Furthermore, AERMOD was used for modeling of NO_x , $PM_{2.5}$ and SO_2 emissions from point and line sources in Halifax, Nova Scotia, Canada in 50 × 50 km^2 domains over 1-hr, monthly and annual periods averages. The evaluation of model performance was done by applying

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statistical parameters and the results showed poor agreement among the observed and simulated results (Gibson et al., 2013). In another study, Cohan et al. (2011) examined the dispersion and predicted concentrations of NO_x and $PM_{2.5}$ in port communities in California using AERMOD. The correlation coefficients for NO_x and $PM_{2.5}$ were 50% and 43%, respectively (Cohan et al., 2011). AERMOD model was also used to simulate the NO_2 and SO_2 concentration in Beilun area. The results demonstrated that the ambient average concentrations of NO_2 and SO_2 were equivalent to 16.7%~58.3% and 26.7%~53.3% of the clean air quality standard respectively. However, concentrations of NO_2 and SO_2 were relatively higher in the upper air of Beilun region. NO_2 and SO_2 gasses in the upper air were a reason for the high potential of acid rain on Beilun region (Hasson et al., 2013).

In order to study the performance of applying emission control devices, AERMOD modeling system was applied for predicting the air pollution in Xuanwei, an important industrial city in China, and it was concluded that AERMOD can be used properly for simulating NO_x and SO_2 emissions (Ma et al., 2013). The emission, transport, dispersion, and concentration analysis of PM emitted from a big industrial complex in Malagueño city, Argentina, were also conducted using AERMOD model. The model was applied to 224 emission sources. The performance of the model was evaluated by comparing the simulated results with the observed Total Suspended Particulate matters (TSP) at two monitoring sites for 62 continuous days in winter. The results showed the impact of the industrial activities on the local PM concentrations, from which stockpiles and unpaved industrial roads were the main emission sources, straightly affecting two of the nearest neighborhoods in the study area (Abril et al., 2016). In another study, AERMOD model was used to predict SO_2, CO, NO_x and PM_{10} emissions impacts at receptors due to calcining processes including handling and storage of raw petroleum coke in Argentina. The observed and predicted ambient pollutants' concentration levels were compared with public health standards. The results revealed that the exposures of the simulated NO_x, CO, PM_{10} and SO_2 concentrations were lower than the air quality standards. However, the PM concentration level was higher than the standard limits (Singh et al., 2015).

The exposures of various pollutants including NH_3, CH_4, CO, CO_2, H_2S, NO_2, N_2O, SO_2 and organic dust were estimated for populations residing in close vicinity to ten poultry concentrated animal feeding operations (CAFOs) located in Central Poland. AERMOD model was used to predict the pollutants' concentrations in order to compute the Hazard Index (HI) for a mixture of chemicals. Results presented that the estimated hazard indexes which were less than unity; therefore there was low potential for adverse health consequences for the surrounding society for the combined mixture of chemicals (Pohl et al., 2016).

A comparative study regarding the health effects of emissions due to vehicles and industries were conducted in China and Pakistan. In this study, ambient CO, NO_2, and SO_2 concentrations were measured and compared with U.S. EPA, WHO and national clean air standards in China and Pakistan (Niaz et al., 2015).

The performances of ISCST-3, AERMOD, and CALPUFF models for NO_x and CO emissions in point, line and area sources were compared together using statistical analysis in Körfez. The results revealed that AERMOD predictions for NO_x concentrations were lower than those predicted by ISCST-3, and CALPUFF models. Meanwhile, CO concentrations predicted by AERMOD were among the concentration levels simulated by CALPUFF and ISCST-3 (Demirarslan and Doğruparmak, 2016).

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The main goal of the present study was to simulate the ambient concentrations and dispersion patterns of NO_2 emitted from stacks and flares in a sour gas refinery located in a complex industrial region with special climatological and topographical conditions and to determine the contribution of the gas refinery in NO_2 emissions using AERMOD dispersion model.

In this study, ambient NO_2 concentrations in a gas refinery situated in Asaluyeh was measured seasonally in 10 receptors in and around the refinery from June 2014 to May 2015. Thereafter, the 1-hr and annual ambient concentrations and dispersion patterns of NO_2 were simulated by applying AERMOD dispersion model in the area of 10×10 km^2. Moreover, the predicted concentrations in receptors were compared with the measured ones using statistical methods. Then, unhealthy zones were determined in the study region using contour plots of the seasonal NO_2 distribution patterns. Finally, the contribution of emissions due to point sources in this refinery was determined.

Materials and Methods

Study Area

Asaluyeh, a sub-city of Kangan, belongs to Boushehr Province, which locates in the southwest of Iran. The desired gas refinery as shown in *Figure 1*, is situated on 27° 30’ to 27° 31’ north latitude and 52° 34’ to 52° 36’ east longitude in Asaluyeh. It was commissioned with a refining 50 million cubic meters per day sour gas and 80,000 barrel per day gas condensate to supply for the Iran’s local strategic demand.

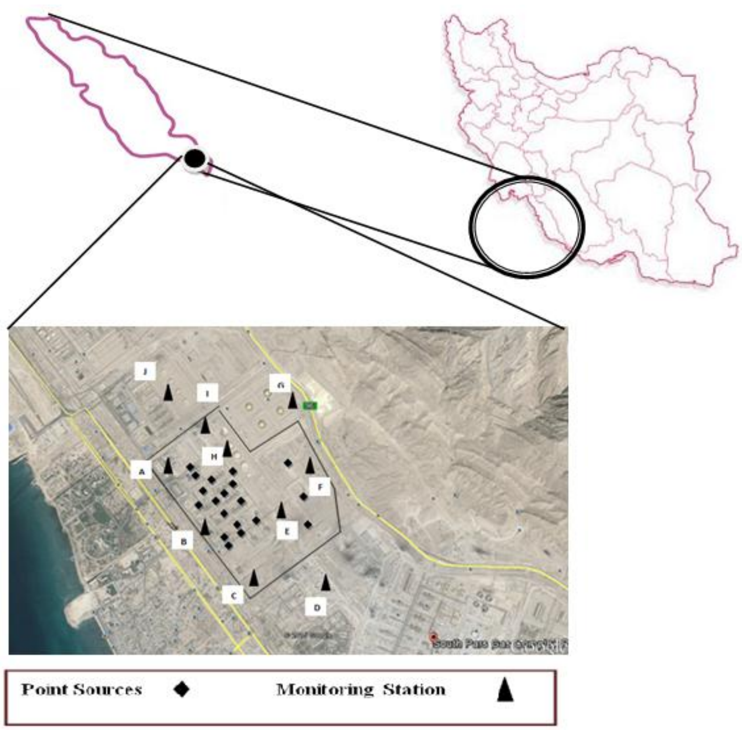

***Figure 1.** The study area, the point sources and monitoring stations*

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Field Measurements

Emission Sources

The point sources in the gas refinery included 13 stacks and six flares. The exit velocity of the flue gas and concentrations of NO_2 emitted from the stacks were measured by a portable emission analyzer (TESTO 350 XL), in four seasons (June 2014 - May 2015) and four times in each season (ASTM, 2011a). The emission rate of NO_2 resulting from the flares was also determined by emission factors according to EPA/AP-42 method.

The required AERMOD inputs data were including emission height (m), flue gas temperature (°C), inner diameter of stack (m), flue gas velocity (m/s), geographical coordinates (X,Y) and elevation above the ground level (m) (*Table 2*). The locations of point sources are illustrated in *Figure 1*. In the current study, the maximum NO_x emission for the continuous operation has been considered. The total NO_2 emission rate from 19 point sources which evaluated according to Tier2 in AERMOD guidelines (US EPA, 2009, 2010a) was 270 g/s.

Table 2. *Characteristics of point sources in the gas refinery*

Sources*	**Cartesian Coordinates**		**Diameter**	**Height**	**Flue Gas Temperature**	**Flue Gas Velocity**
	X (m)	**Y (m)**	**(m)**	**(m)**	**(°C)**	**(m/s)**
BO-1	0	0	3	42.7	160.5	6.1
BO-2	-17	-10	3	42.7	154	5.2
BO-3	-33	-26	3	42.7	159	5.2
BO-4	-49	-35	3	42.7	154	5.1
GTG-1	-183	145	4	30	550	32
GTG-2	-167	130	4	30	550	32
GTG-3	-153	111	4	30	550	32
GTG-4	-139	94	4	30	550	32
GTC-1	311	-110	3.15	17.3	520	30
GTC-2	325	-129	3.15	17.3	520	30
GTC-3	339	-129	3.15	17.3	520	30
X-1	129	-266	2.1	117	592	10
X-2	31	-138	2.1	117	500	10
F-1**	998	221	0.48	142.8	800	60
F-2**	1057	474	0.48	142.8	800	60

* BO: Boiler, GTG: Gas Turbine Generator, X: Incinerator, GTC: Gas Turbine Compressor, F: Flare
**According to Iowa Procedure the modified diameter and height were used for flares.

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Ambient NO_2 Concentrations

Measurements of 1-hr average of ambient NO_2 concentrations were performed using a mobile device (LSI-Lastem Babuc A) in four seasons and four times in each season from June 2014 to May 2015 in 10 receptors (ASTM, 2011b). The locations of receptors from reference point (BO-1) have been presented in *Table 3*.

Table 3. *Cartesian coordinates of the receptors*

Receptor	**Cartesian Coordinates**	
	X (m)	**Y(m)**
A	402	-478
B	-127	-141
C	-286	712
D	-460	667
E	-201	982
F	-13	846
G	55	799
H	594	343
I	904	-4
J	691	-281

Meteorological Data

All Surface and upper-air weather data (wind speed, wind direction, temperature, cloud cover, dew point temperature, pressure at sea level, rainfall and humidity, solar radiation) in eight time steps (0:00, 3:00, 6:00, 9:00,12:00, 15:00, 18:00, 21:00) in a day were provided as meteorological inputs for AERMOD from June 2014 to May 2015 (IRIMO, 2015).

The annual wind rose was produced based on data received from Asaluyeh Weather Station (*Figure 2*). The prevailing wind direction in this station was from NW to SE in all seasons. The AERMET was employed to simulate the climatically conditions using the values of Bowen ratio, surface roughness length and Albedo parameters based on the types of the surrounding vegetation and land use in the study area. The values of these parameters have been presented in *Table 1*.

Table 1. *The surface specifications according to meteorological data*

Sector Number	**Beginning Direction (Degree)**	**Ending Direction (Degree)**	**Albedo**	**Bowen Ratio**	**Roughness Length (m)**
1	0	150	0.28	6	0.3
2	150	300	0.14	0.1	0.0001
3	300	360	0.28	6	0.3

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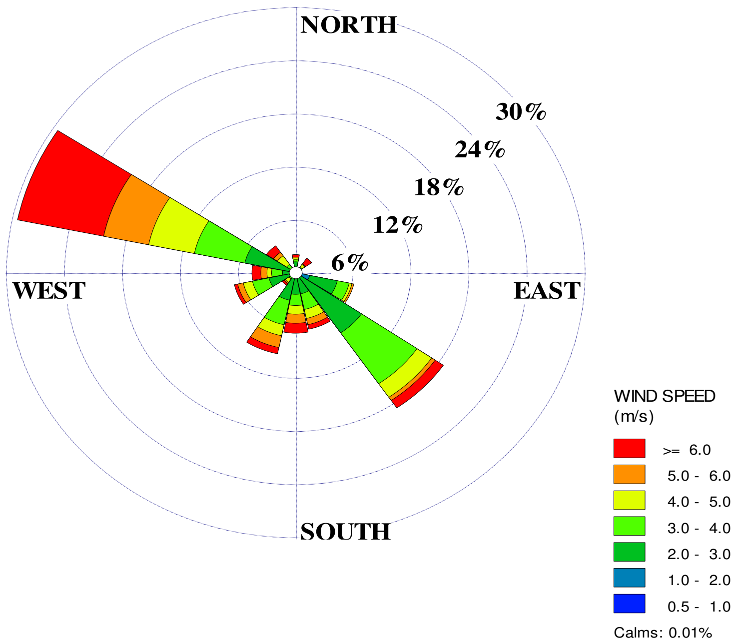

Figure 2. *The annual wind rose in the study area*

Elevation Data

In order to predict the NO_2 concentrations at receptors, elevation data was required as model input. In this study, the required digital elevation files were provided from Iran National Cartographic Center. The 3D view of topography in the study region produced by GIS with a resolution of 90 m has been shown in *Figure 3*.

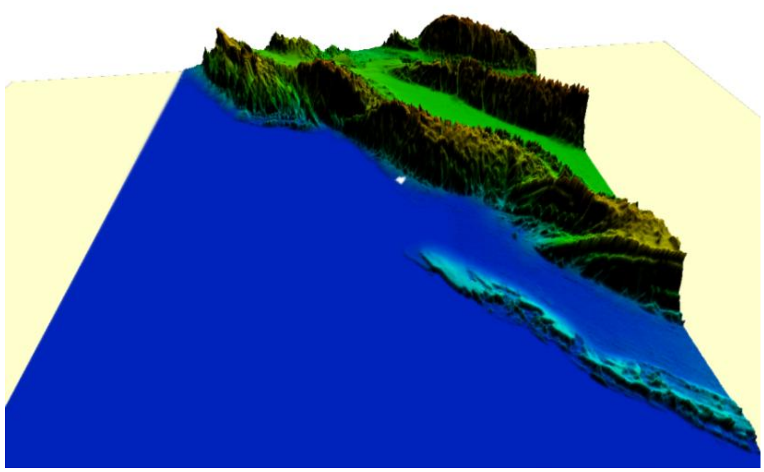

Figure 3. *The 3D view of topography in the study region*

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AERMOD Modeling System

AERMOD dispersion model developed by the U.S. EPA is a steady-state Gaussian model for predicting air pollutants' concentrations and dispersion patterns due to various emission sources. AERMOD model consists of three sections: Meteorological Preprocessor (AERMET), Terrain Preprocessor (AERMAP) and AERMOD Gaussian Plume Model (US EPA, 2004a, 2004b, 2009). It is necessary to provide all emission rates due to various sources (point, line, area, and volume sources) and the meteorological and topographical data to predict pollutants' concentrations and dispersion patterns in rural and urban areas, calculating building downwash effect, terrain adjustment, etc. at short-range (up to 50 km) (Cimorelli et al., 2005; Perry et al., 2005). AERMOD makes distribution estimations by utilizing the meteorological characteristics of the study region, including stack diameter and height; stack temperature, flue gas velocity, wind direction and wind speed (Mokhtar et al., 2014; Huertas et al., 2014; Stein et al., 2007).

In this study, simulations for NO_2 ambient concentrations and dispersion patterns were performed for the total study area of 100 km^2, where a grid of 50 m × 50 m was built.

Evaluation of AERMOD Performance

In order to evaluate the model performance, the results of field measurement and model simulation were compared together using statistical methods. Statistical Parameters suggested by U.S. EPA (U.S. EPA, 2003, 2005) including: "correlation coefficient (COR) ", Normal Mean Bias (NMB)", "Normal Mean Error (NME), "fractional bias (FB)," and also "Index of Agreement (IOA)" (Luhar, 2003) were applied (*Equations 1 ~ 5*). According to *Eq. (1)*, parameter COR indicates the relationship between the Simulated and the measured results. The more the value of COR is closer to 1, the performance of the model is more satisfactory.

$$COR = \frac{\sum_{i=1}^{N}(S_i-\bar{S})(M-\bar{M})}{\left(\sum_{i=1}^{N}(S-\bar{S})^2\sum_{i=1}^{N}(M_i-\bar{M})^2\right)^{1/2}} \quad \text{(Eq. 1)}$$

NMB and NME parameters were applied to evaluate the model performance for simulating NO_2 concentrations. The variation ranges for COR, NMB and NME were (-1 ~ + 1), (-1 ~ + ∞), (0 ~ + ∞), respectively (U.S. EPA, 2003).

$$NMB = \frac{\sum_{i=1}^{N}(S_i-M_i)}{\sum_{i=1}^{N}M_i} \times 100 \quad \text{(Eq. 2)}$$

$$NME = \frac{\sum_{i=1}^{N}|S_i-M_i|}{\sum_{i=1}^{N}M_i} \times 100 \quad \text{(Eq. 3)}$$

FB indicates the tendency of the model to predict values more or less than the measured concentrations in this study (Olesen, 2001). The acceptable value for FB was less than 0.3 (Ghannam and El-Fadel, 2013).

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$$FB = \frac{(\overline{M_i} - \overline{S_i})}{0.5(\overline{M_i} + \overline{S_i})} \quad \text{(Eq. 4)}$$

IOA is sensitive to differences between the measured and simulated results means as well as to certain changes in proportionality (Luhar, 2003). The variation range for IOA was (0,1).

$$IOA = 1 - \left[\frac{\sum_1^N (S-M)^2}{\sum_1^n (|S-\overline{M}| + |M-\overline{M}|)^2}\right] \quad \text{(Eq. 5)}$$

Where, S_i: simulated concentrations, M_i: measured concentrations, $\overline{S}$: average simulated concentrations, $\overline{M}$: average measured concentrations, N: the total number of measurements.

Results and Discussion

Experimental Results

The 1-hr averages of ambient NO_2 concentrations in 10 receptors in four seasons from June 2014 to May 2015 have been illustrated in *Figure 4*. For all receptors except B in summer 2014, the measured ambient NO_2 concentrations were lower than the clean air U.S. EPA standard for 1-hr ambient concentration levels. However, in all seasons the simulated ambient NO_2 concentrations in all receptors were lower than the standard limit (200$\mu g/m^3$). Based on the safety regulation for hourly NO_2 concentrations (200 $\mu g/m^3$) published by the WHO Guideline, NO_2 emissions from the gas refinery showed no significant health effects on personnel. However, the average annual concentration was 87 $\mu g/m^3$ which was higher than the WHO standard limit (40 $\mu g/m^3$) and they have not exceeded the U.S. EPA standard limits (100 $\mu g/m^3$) (WHO, 2017; U.S. EPA, 2010b).

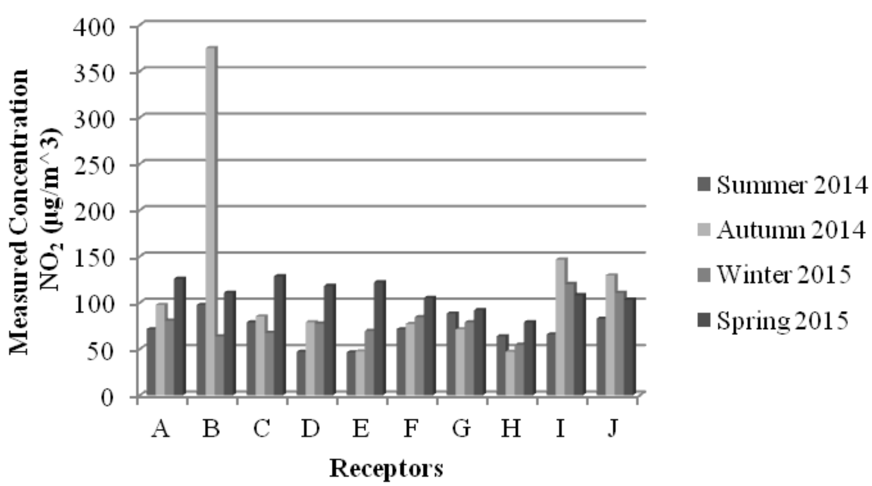

Figure 4. *Ambient NO_2 concentrations in 10 receptors at 1-hr average period*

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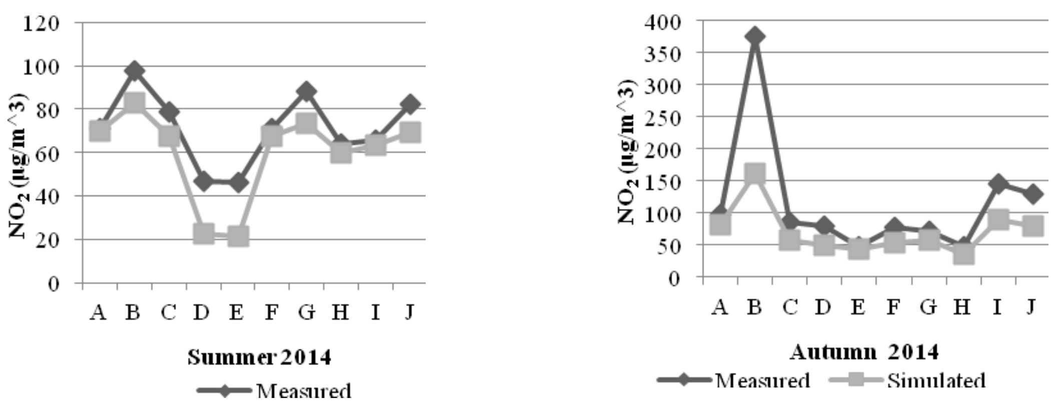
The 1-hr averages of ambient NO_2 concentrations measured in 10 receptors varied from 46.4 to 97.6 $\mu g/m^3$ in summer 2014, from 47 to 375 $\mu g/m^3$ in autumn 2014, from 54.5 to 120.2 $\mu g/m^3$ in winter 2015 and from 78.9 to 125.8 $\mu g/m^3$ in spring 2015. As a result, the highest observed NO_2 ambient concentration was measured in autumn and the lowest amount was measured in summer for 1-hr average period. However, in all seasons the predicted ambient SO_2 concentrations in all receptors were lower than the US EPA clean air standard levels except for B monitoring stations in autumn 2014, due to the direction of prevailing wind (from NW to SE) and the neighboring refineries.

The simulated results also showed that the variations of NO_2 concentrations were ranged from 21.2 to 83.03 $\mu g/m^3$ in summer 2014, from 35.7 to 162 $\mu g/m^3$ in autumn 2014, from 34.5 to 87 $\mu g/m^3$ in winter 2015, and from 73 to 114.5 $\mu g/m^3$ in spring 2015, which presented the highest concentration in the autumn and the lowest value in the summer. Meanwhile, in all seasons the simulated ambient NO_2 concentrations in all receptors were lower than the clean air standard levels.

A comparison among the results of measured 1-hr average NO_2 concentrations and the simulated values for 10 monitoring stations (receptors) in four seasons in the study area is presented in *Figure 5*. As shown, the simulated ambient NO_2 concentrations were less than the measured ones for all receptors. The variations among the simulated and measured ambient NO_2 concentrations in D, I and J receptors were higher than those in other receptors. These receptors were situated on the border of the study area with other refineries and it might be resulted from emissions due to other neighboring refineries and other industries. The observed and simulated results were in good agreement. The values and locations of maximum simulated ambient NO_2 concentrations at 1-hr and annual averages are presented in *Table 4*.



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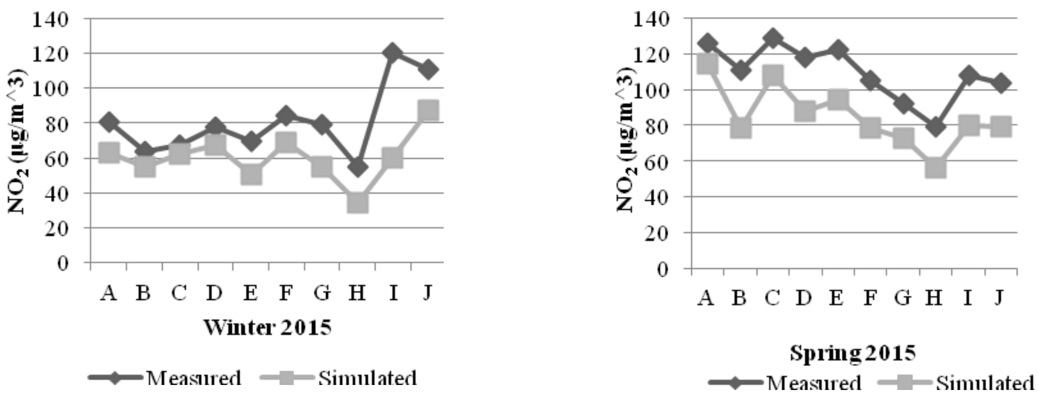
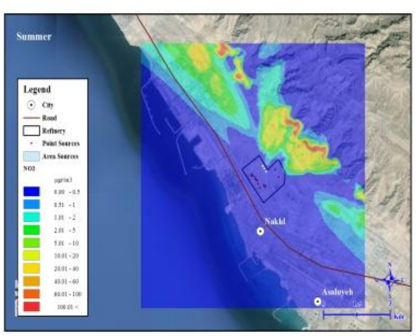


Figure 5. *Comparison among measured and simulated 1-hr averages of ambient NO_2 concentrations for 10 receptors*

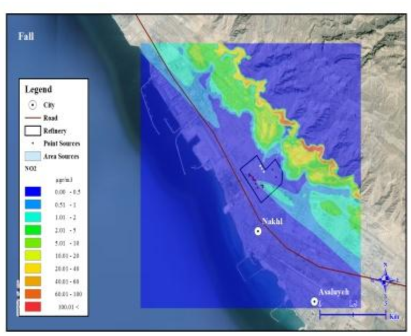
Table 4. *Maximum simulated amounts of ambient NO_2 concentrations based on the average periods in a 10×10 km^2 domain*

Time Scale	Max. Concentration (µg/m³)	hour	day	month	X (m)	Y (m)	Z (m)
1-hr	10544.1	19	21	11	1650	2050	1.5
Annual	217.4	-	-	-	2550	1050	1.5

The NO_2 distribution maps based on annual simulated results among the four seasons are presented in *Figure 6*. In this figure, the areas with intensive color were more affected by NO_2 emissions and defined as unhealthy zones. As shown in the figure, the unhealthy zones were located in the right part of the simulating domain.



a) Summer 2014



b) Autumn 2014

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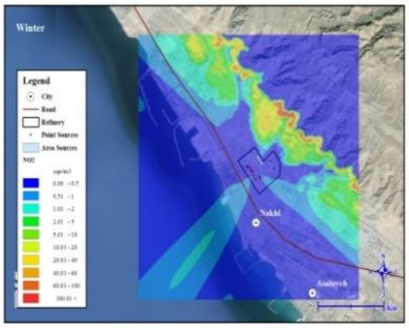
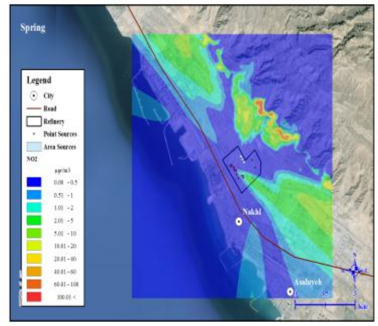


c) Winter 2015



d) Spring 2015

Figure 6. *NO_2 distribution maps for the simulated results in the study area*

1-hr maximum concentrations of the simulated and measured values were used for the statistical analysis for evaluating the model performance. The values of correlation coefficients (COR) for NO_2 concentrations were 0.89 in summer 2014, about 0.87 in autumn 2014, about 0.67 in winter 2015 and about 0.73 in spring 2015. The results are shown in *Table 5*. As a result, there were acceptable ranges for statistical parameters and the model simulation indicated that the simulated concentrations were underestimated in compare to the measured ones.

Table 5. *The results of statistical analysis*

Season	**COR (0,1)**	**NMB ≤%15**	**NME ≤%30**	**IOA (0,1)**	**FB <0.3**
Summer (2014)	0.89	-16	16	0.86	-0.017
Autumn (2014)	0.87	-38	28	0.70	-0.047
Winter (2015)	0.67	-21	25	0.61	-0.023
Spring (2015)	0.73	-22	22	0.63	-0.025

Since there are some limitations for applying AERMOD modeling system, the simulation results may not be quite accurate. The limitations are such as: lack of module for considering NO_2 deposition reactions; high sensitivity of AERMOD to various time scales (Zou et al., 2010; Bhardwaj, 2005); lack of considering the dynamic emission rates (U.S. EPA, 2009); lack of considering the substantial time intervals for wind velocities less than 1 m/s; errors due to monitoring devices (Drew et al., 2007); complexity in topographical structure of the study area (El-Fadel et al., 2009), coastal evaporation, and the geographical effects (Yao et al., 2011).

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A comparison made among the measured and predicted ambient NO_2 concentrations. The results indicated that the predicted concentrations were underestimated by a factor of 20 % in comparison to the measured ones which indicated the contribution of other sources including mobile sources and the neighboring gas refineries and other industries.

Conclusions

In the present study, ambient NO_2 concentrations in a gas refinery located in Asaluyeh was measured in 10 receptors in and around the refinery and the 1-hr and annual ambient NO_2 concentrations and dispersion patterns were simulated by AERMOD model in 10×10 km^2 domain. Then the unhealthy zones were determined in the study area using contour plots of the seasonal NO_2 distribution patterns. It was found that the hourly observed and predicted concentrations of NO_2 were lower than the U.S. EPA clean air standard except in receptor B in autumn 2014. However, there was no health risk due to NO_2 emissions for short time (1-hr) exposure in the gas refinery. Furthermore, a comparison made among the measured and the simulated NO_2 concentrations in 10 receptors. The results indicated the performance of the modeling was quite satisfactory with little disagreement in the values of predicted results. The discrepancy between the average simulated and measured concentrations varied from 2% to 56%; however the average predicted concentrations were underestimated by a factor of 20 % in comparison to the measured ones.

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AGRICULTURAL WATER-SAVING POTENTIAL FOR GUANZHONG IRRIGATION AREAS UNDER DIFFERENT GUARANTEED RATES OF PRECIPITATION

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Abstract. The Agricultural Water-saving Potential (AWP) has important influences on water-saving technology development, regional irrigation water management, agricultural production and water ecological balance protection. The study aims to design a reasonable methodology to calculate the AWP and analysis the AWP of Guanzhong irrigation area, in Weihe River Basin of China. The AWP was divided into resource-based AWP and efficiency-based AWP. The former was calculated according to the coefficient of water transmission in canals and irrigation in the field. The latter was estimated on the basis of the field evapotranspiration. The guaranteed rates of precipitation were chosen as the hydrologic conditions. Under the guaranteed rates of 25%, 50%, 75%, 90% and 95%, the annual total AWPs of the Guanzhong irrigation areas reached 30.69×106 m^3, 111.67×106 m^3, 11.75×106 m^3, 12.52×106 m^3, and 14.76×106 m^3 respectively. The efficiency-based AWP occupied a proportion of more than 50% in each condition, which indicated that the improvement of water utilization efficiency should be in preference to improvement of water conveyance efficiency in technology innovation. Summer maize, fruit tree and cotton made the largest contribution to efficiency-based AWP while there was not a big difference among the 3 crops in various irrigated areas when it came to the AWP per unit area, and their average values were 9.4, 6.3 and 4.3 mm respectively. Then the study comes up with measures and suggestions to improve the AWP according to the planting structures and features of the 9 irrigated areas. This method takes into account the water consumption during the processes of irrigation and crop growth. It can provide the support for water saving agriculture. The accurate calculation of water dissipation coefficient is the key to the efficiency of the AWP method, and the stability of the measurement coefficient will be improved in the future.
Keywords: *resource-based water-saving, efficiency-based water-saving, Weihe River Basin, precipitation insurance rate, water utilization efficiency*

Introduction

Both water resources per capita and water resources per area are low in China (Yang et al., 2012). Particularly, the distribution of water resources in the country is seriously uneven, with large amount of freshwater resources concentrating in the south. By contrast, the total amount of water resources in the north of the Yangtze River (covering the Haihe River Basin, Huaihe River Basin, Yellow River Basin, and Northwest and Northeast China) is about 546 billion m^3, accounting for only 19% of the national total (Hu et al., 2010). According to the International Water Management Institute (IWMI), water shortage in most countries and regions in the world is correlated to agriculture. The research (Yang et al., 2012) indicates that, if irrigation water used in global food production could be reduced by 10%, people’s water demands for all other purposes

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wouldbe met. Accounting for the largest part of the total water use, agricultural water is the most important factor in regional water balance and sustainable utilization of water resources (Belder et al., 2004). Yang and Tian (2009) and Fan et al. (2010) studied the relation between agricultural irrigation water and surface runoff in typical water basins in North China (NC), and the results show that agricultural production may be the major reason for decrease in surface runoff and exhaustion of rivers in most northern river basins. Moreover, groundwater overdraft has resulted in the generation of the world's largest underground cone of depression, sharp drop of underground water level, river blanking, and continuously decreasing water flow in NC; these phenomena indicate that water resources have become increasingly scarce in semi-humid and semi-arid regions and arid regions in China (Moiwo et al., 2009; Zhang et al., 2013; Yin et al., 2016).

Currently, the overall utilization efficiency of irrigation water in China is relatively low, and the biggest water loss is percolation and evapotranspiration in the process of water transmission (Wang et al., 2012a). In addition, farmlands are still the key link of agricultural water saving. The core of agricultural water saving is to reduce and effectively control water use throughout three processes of water transmission and loss in farmlands, which are respectively soil water evaporation, plant transpiration, and soil water percolation (Nair et al., 2013). Because percolating water could be reused through nearby water transfer, the most important factors influencing water consumption on the scale of irrigation area are soil water evaporation and plant transpiration (Yan et al., 2015).

After years of expansion, studies on "agricultural water-saving" have covered all links of agricultural production, and one of the important contents of these studies is to improve the overall utilization efficiency of agricultural water resources (Kifle and Gebretsadikan, 2016). In terms of scale, agricultural water-saving has gone far beyond the concept of "farmland" and was gradually employed 3 scales: plant – farm – area (irrigation area in most cases) (Yang et al., 2012; Nair et al., 2013). The studies are aimed to develop theories and technologies for realizing agricultural water-saving, and strategies on the utilization of water resources. In the next place, except for agricultural water-saving at the technical level, water-saving at the management level is receiving more and more attention as well (Huffaker and Whittlesey, 2003; Hu et al., 2010; Yan et al., 2015; Damerau et al., 2016). In this respect, Agriculture Water-saving Potential, AWP, is an important index for water resources management, which can be used to evaluate the amount of water-saving in a certain area (Horst et al., 2005; Yan et al., 2015). Calculating AWP on the scale of irrigation area has certain practical meaning in both agricultural water-saving and water resource security (Zhang and Guo, 2016).

The concept of "Water-saving Potential" was first proposed in researches on urban and residential water management. Dixon et al. (1999) evaluated cities' water-saving potentials in studying the effect of reclaimed water reuse and rainwater utilization on cities' water-saving capacity. Based on investigations, Yurdusev and Kumanlıoğlu (2008) divided domestic water into different categories of water used respectively in washroom, toilet, bathroom, kitchen, washing machine, dishwasher, and for watering flowers outside, washing cars, etc., and defined water-saving potential as the sum of water-saving amounts achieved under all the categories. By using the concept of agricultural water-saving for reference, researches on estimation of AWP play a more and more important role in water resources management. Karimov et al. (2012) calculated the water-saving potential of Fergana Valley using the "water accounting procedures", which are based on the principle of water balance. According to the

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method, they first analyzed the using condition of the water resources in the study area, then worked out the amount of ineffective water consumption, namely the water-saving potential, and finally proposed some measures to reduce the ineffective water consumption, such as evapotranspiration reduction and depression detention, and to increase effective utilization, such as expansion of planting area. With the geo-information system (GIS) and remote sensing (RS) technologies being widely used in the calculation of water resources potential, AWP on a regional scale has been gradually introduced to various studies. Horst et al. (2005) analyzed the water saving potential through the application efficiency, the distribution uniformity and total applied irrigation depths with furrow irrigation in Fergana, Aral Sea basin, to assess the potential for improving the performance of furrow irrigation in the central part of the Fergana Valley, Uzbekistan. Yan et al. (2015) project the results of such experiments for winter wheat, maize, and cotton to basin scale to assess their potential in restoring sustainable water consumption. The study employed the remote sensing model-ETWatch (Wu et al., 2012) for calculating the crop water comsuption in planting area, and found that the mulching, which would reduce the over-consumption by 25%, is the most promising option for farmers in Hai River Basin.

China has great AWP (Yang et al., 2009; Damerau et al., 2016), and the utilization efficiency of agricultural water resources in China will be finally improved by relying on reasonable irrigation methods, adopting advanced irrigation technologies, establishing perfect irrigation managing system and institution, and improving biological attributes of fields and crops, so as to effectively alleviate the pressure from water resource shortage (Yan et al., 2015). The Guanzhong region in Weihe River Basin serve as the bond and bridge connecting the western and eastern regions in North China. The Guangzhong region includes nine main irrigation areas, which are the major grain-producing areas in this region. In recent years, the economic and social development in this region has been limited by the serious situation of water shortage. Although the local government devotes itself to adjusting water utilization structure, agriculture is always the largest source of water use (Tang et al., 2016). The total amount of water resources in the Weihe River Basin is 10.683 billion m^3, with total surface water resources being 9.012 billion m^3. By 2015, the overall utilization ratio of surface water resources in the river basin reached to near 30%, but the potential was low, with no potential left in some tributaries; the total amount of groundwater resources is 4.506 billion m^3, but the exploitation rate has exceeded 62%, indicating this region is a seriously overdraft area (Ministry of Water resources of the P. R. China 2015). The water deficit is expected to be more than 2.4 billion m^3 by 2020, and more than 3.0 billion m^3 by 2030 (Tang et al., 2016). Meanwhile, the productivity of agricultural water resources in the irrigation areas is low, being 1 $kg \cdot m^{-3}$ on average, much lower than that other agriculturally advanced countries (Tang et al., 2014a). The causes for the low water resource efficiency mainly include lack of water-saving hi-tech applications, low lining rate of water conveyance canals, and insufficient researches on the irrigation areas' actual carrying capacity of water resources (Tang et al., 2014b). So, there is a great potential in exploiting water resources in this region, but the question is: how much is the potential after applying agronomic measures and water-saving irrigation technologies? Reasonable analysis of AWP has great practical and referential significance in developing agricultural water-saving and making water-saving policies and water resources utilizing plans.

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Considering that water dissipation mainly occurs in two different processes: water transmission (Tang et al., 2014a) and field evapotranspiration (Wu et al., 2012; Pereira, 2017), water consumption could be influenced directly by changing the two links (Wang et al., 2011). The two processes were discussed separately in this study. The amount of water that can be saved in the process of water transmission is denoted as resource-based saving amount, and the amount of water that can be saved in the field is denoted as efficiency-based saving amount (Wang et al., 2011). In addition, at different precipitation levels, the amounts of irrigation water are different from each other (Bennett et al., 2014; Siderius et al., 2015), so precipitation has to be considered in discussions on water-saving potential and the potential shall be estimated at different Guaranteed Rate of Precipitation (GRP) (Zhang et al., 2007).

By selecting the climate condition (precipitation), in which the GRP was respectively 25%, 50%, 75%, and 90%, the water-saving potentials of the main irrigation areas in the Guanzhong region were analyzed. The water-saving potentials of main crops (winter wheat, summer corn, cotton, fruit tree, cole) in the irrigation areas under the extreme drought condition, in which the GRP was 95%, were analyzed emphatically. Finally, some suggestions on agricultural water-saving modes were proposed according to the current water-saving technologies and the features of planting structure in the nine irrigation areas. This study aims to establish a method to estimate water saving potential, as a theoretical basis for effective utilization of water resources in the irrigation areas and comprehensive development and utilization of water resources in the Weihe River Basin.

Materials and Methods

The Study Area

Guanzhong irrigation areas are located in Shaanxi Province, and involve 5 prefecture-level cities: Baoji, Xi'an, Xianyang, Tongchuan, and Weinan. Agricultural irrigation water in Shaanxi Province concentrates in the areas. Including 9 major irrigation areas and covering a total area of 22,000 km^2, Guanzhong irrigation areas are mainly distributed in the Weihe Valley and valleys of its tributaries (*Fig. 1*). The irrigation areas are located in the continental monsoon climate zone. with a multi-year average precipitation of 600 mm. The mean annual temperature varies from 7.2 °C to 15.2 °C (Wu and Sun, 2016). Precipitation concentrates in July and August, and the precipitation in the two months accounts for nearly 50% of the annual total (Wu et al., 2017). The irrigation areas enjoy abundant sunshine, with a multi-year average sunshine duration of 1,900-2,400 h. Annual actural evapotranspiration in the irrigation areas is 900-1200 mm (Wu et al., 2017). The difference between annual evapotranspiration and precipitation is relatively great, with the highest in summer and lowest in winter and the difference in spring is higher than that in autumn. Defined as semi-arid and semi-humid irrigated agricultural areas in warm temperature zone, Guanzhong irrigation areas are suitable for planting a variety of crops. Guanzhong irrigation areas account for 1/3 of the total grain production in Shaanxi Province (Wang et al., 2011). In addition, vegetables, fruits, and other commercial crops are widely grown in the areas as well, and multiple-cropping index is high. The main food crops in the irrigation areas include wheat, maize, and beans, and the commercial crops include cotton, oil plants, fruits, vegetables, etc. Fruit-planting areas

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are mainly located in Baojixia, Luohuiqu, Yangmaowan, Shibaochuan, Taoqupo irrigation areas (Wang et al., 2012b; Yin et al., 2013).

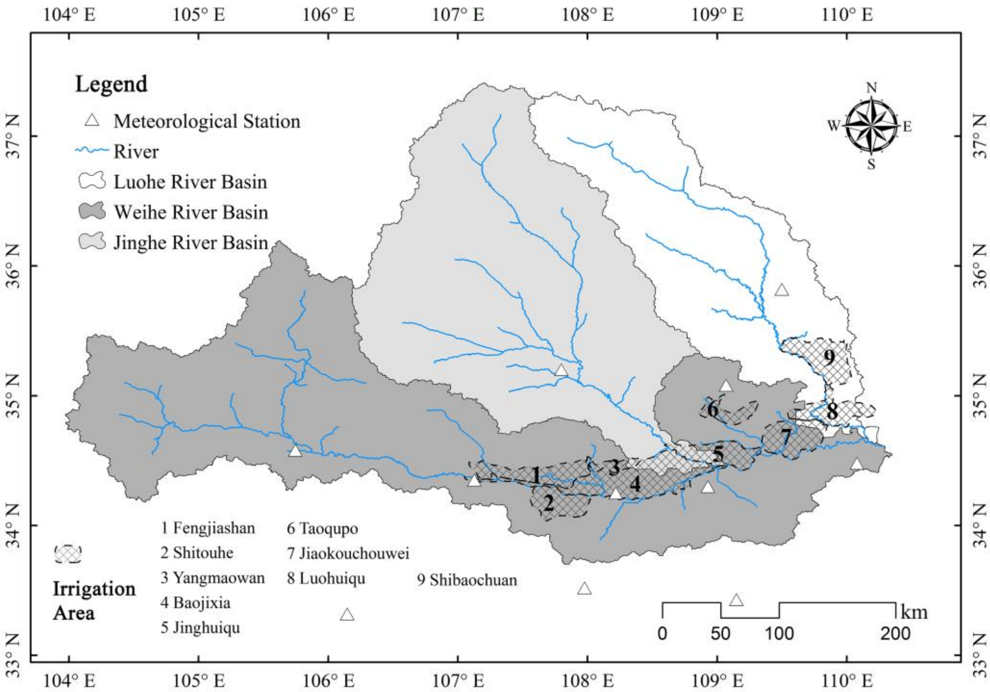

Figure 1. *Map of the 9 irrigation areas in Guanzhong area*

Over the years, the average annual amount of diverted water in the irrigation areas is 1.787 billion m^3, 82.65% of which is diverted by means of ground channel system; the other water is drawn from on-site wells (Yin et al., 2013). The irrigation water for the irrigation areas is mainly taken from Jinghe River, Luohe River, and Weihe River, but the runoff of these rivers is distributed unevenly throughout the year, with the runoff from June to October accounting for 50-70% of the annual runoff (Wang et al., 2011).

Data sources

Meteorological and hydrological data used in this study includes precipitation, temperature, relative humidity, wind speed, sunshine duration, air pressure, vapor pressure, and evapotranspiration data recorded by a number of meteorological stations in the Guanzhong region over 35 years (1981-2015) and provided by China Meteorological Data Sharing Service System (http://data.cma.cn/) (the distribution of the meteorological stations is shown in *Fig. 1*). Other data includes social and economic data (mainly about agricultural water and crop structure, such as the area of main crops in Guanzhong irrigation areas, and irrigation water use), which was collected from statistical yearbooks of Shaanxi Province and official reports on water resources of the Weihe River Basin. After collecting natural and social original data, spatial interpolation method was then used to obtain the mean value of Guanzhong irrigation areas.

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Methodology

Theoretical AWP

In the study, we divided AWP into two types, namely resource-based water-saving potential and efficiency-based water-saving potential. The resource-based water-saving potential refers to the saving amount that is achieved by reforming or adjusting water supply to reduce water evapotranspiration and deep percolation; the efficiency-based water-saving potential refers to the saving amount that is achieved by controlling the soil water required for crop growth. The total water-saving potential is the sum of resource-based water-saving potential, W_r, and efficiency-based water-saving potential, W_e, as shown in (Eq.1):

$$W = W_r + W_e \quad \text{(Eq.1)}$$

where, W represents the water-saving potential of an irrigation area, and the subscripts indicate the types of water-saving potential.

The equation for calculating the maximum water-saving potential of a region, $W_{r,m}$ is written as:

$$W_{r,m} = D_c + D_f = (1-\xi_c)I_g + \xi_f(1-\xi_c)I_g = (1+\xi_f)(1-\xi_c)I_g \quad \text{(Eq.2)}$$

where, D respectively represent water loss in the process of water transmission by channels (D_c) and deep percolation in the field (D_f), which can be calculated by using the gross irrigation water amount I_g, the coefficient of water transmission by channels ξ_c, and the coefficient of irrigation in the field ξ_f, From being drawn from water sources to being assimilated by crops, irrigation water is first converted to soil water after being transported to farmlands through channels, and then used by crops through transpiration to generate biomass. Meanwhile, some of irrigation water would lose in forms of soil evaporation, deep percolation, and surface runoff. Therefore, the actual amount of irrigation water needed by crops is much higher than the net irrigation water demand.

The resource-based water-saving coefficient ξ_r is the ratio of resource-based water-saving amount to the gross irrigation water amount, written as:

$$\xi_r = W_{r,m} / I_g = (1+\xi_r)(1- \xi_r) \quad \text{(Eq.3)}$$

The resource-based water utilization coefficient, ζ_r, is written as:

$$\zeta_r = 1- \xi_r = 1- (1+\xi_r)(1- \xi_r) \quad \text{(Eq.4)}$$

If the coefficient of water transmission by channels and the coefficient of irrigation in the field are known, the resource-based water utilization coefficient could be calculated.

Efficiency-based water saving includes two aspects: reducing ineffective water consumption in the growing process of crops, and reducing water consumption by adjusting crops' water consumption process based on crops' physiological characteristics. Thus, efficiency-based water-saving potential includes the potential from reduction of ineffective water consumption and the potential from reduction of

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crop evapotranspiration. The equation for calculating efficiency-based water-saving amount is written as:

$$W_{e,m} = ET_{ine} + \varphi_1 (ET - ET_{ine}) = \varphi_2 ET + \varphi_1 (ET - \varphi_2 ET) = ET(\varphi_1 + \varphi_2 - \varphi_1 \varphi_2) \quad \text{(Eq.5)}$$

where, E_{ine} represents the ineffective water consumption in the field, which is mainly the reduced evaporation from soil and can be expressed as a percentage of crop water consumption (evapotranspiration, ET); φ_1 represents the percentage of water consumption reduction in farmlands achieved by adjusting crops' physiological process; φ_2 represents the percentage of water consumption reduction in farmlands achieved by adopting covering technologies and other agronomic measures.

The efficiency-based water-saving coefficient ξ_e is the ratio of the water-saving amount to evapotranspiration, written as:

$$\xi_e = W_{e,m} / ET = (\varphi_1 + \varphi_2 - \varphi_1 \varphi_2) \quad \text{(Eq.6)}$$

The efficiency-based water utilization coefficient ζ_e is written as:

$$\zeta_e = 1 - \xi_e = 1 - (\varphi_1 + \varphi_2 - \varphi_1 \varphi_2) \quad \text{(Eq.7)}$$

If the evapotranspiration percentage under the condition of less land coverage and the percentage of reduction achieved by sacrificing some yield are known, the efficiency-based water utilization coefficient could be calculated.

Actual AWP

The following assumptions are made in the analysis of maximum water-saving potential of the irrigation areas. For resource-based water-saving potential, assume the maximum utilization coefficient of irrigation water is 1 (the utilization coefficient of channel water is 1, and the utilization coefficient of field water is 1). For efficiency-based water-saving potential, assume the current water consumption of crops is the maximum water consumption. In practice, the utilization coefficient of irrigation water is generally less than 1. With respect to efficiency-based water saving, though crop water consumption could be reduced by using covering technologies and other measures and optimal irrigation water amount and other variables could be determined by water generation function and efficiency curve, the irrigation water for the irrigation areas is relatively less in actual production and can hardly meet crops' water demand, thus making the evapotranspiration in actual water consumption less than the maximum one.

The equation for calculating actual potential water saving amount W_p of the irrigation areas is written as:

$$W_p = I_d / \zeta_2 - I_d / \zeta_1 \quad \text{(Eq.8)}$$

where, ζ_1 and ζ_2 respectively represent the resource-based water utilization coefficients before and after applying water-saving technologies, equal to the product of the utilization coefficient of channel water, ξ_c, and the utilization coefficient of field water, ξ_f, I_d represents the net irrigation water demand of crops in the irrigation areas, which

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depends on crops' evapotranspiration (E), precipitation (P), effective utilization coefficient of precipitation α, and crop planting area A:

$$I_d = \sum_{i=1}^{n} A_i (E_i - P\alpha - G) \quad \text{(Eq.9)}$$

where, the subscript, i, represents crop variety; A_i and E_i respectively represent the planting area and evapotranspiration of the ith crop; P and α are respectively the precipitation and the effective utilization coefficient of precipitation in the region. Because the groundwater level in the Guanzhong region is relatively deep, the influence of groundwater contribution G on the crops could be neglected and the value could be approximated as 0.

The equation for calculating the resource-based water utilization coefficient, namely the utilization coefficient of irrigation water, is written as:

$$\zeta_1 = \xi_{c,1} \times \xi_{f,1},\ \zeta_2 = \xi_{c,2} \times \xi_{f,2} \quad \text{(Eq.10)}$$

The utilization coefficient of channel water could be increased by canal lining and other measures, and the utilization coefficient of field water could be increased by land leveling, ridged field improvement, and applications of water-saving irrigation technologies. If the irrigation area of the irrigation areas and irrigation water (I) stay unchanged, and the utilization coefficient of field water is improved to $\xi_{f,2}$, then the resource-based water-saving amount is:

$$W_{r,p} = I(\zeta_2 - \zeta_1) = I \times \xi_{r,p} \quad \text{(Eq.11)}$$

where $\xi_{r,p}$ represents the resource-based water-saving coefficient, which can be written as:

$$\xi_{r,p} = \xi_c \times \xi_{f,2} - \xi_c \times \xi_{f,1} \quad \text{(Eq.12)}$$

Efficiency-based water saving mainly includes two aspects: reducing ineffective water consumption in the growing process of crops, and reducing water consumption by adjusting crops' water consumption process based on crops' physiological characteristics. The equation for calculating the efficiency-based water-saving potential, $W_{e,p}$, is written as:

$$W_{e,p} = \Delta ET / \zeta_2 = ET_b(\varphi_1 + \varphi_2 - \varphi_1\varphi_2)/\zeta_2 \quad \text{(Eq.13)}$$

where, ΔET is the reduction in crop evapotranspiration; ET_b is the water consumption of crops in the base year, which can be obtained by using the Penman-Monteith equation, recommended by FAO56 and various crop coefficients (Pereira et al., 2015).

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Results

AWP with different GRP

Resource-based water-saving amount is reduced water percolation and evaporation, which are saved by implementing water-saving projects. After implementing water-saving projects in Guanzhong irrigation areas, the utilization coefficient of channel water and the utilization coefficient of irrigation water were increased to 0.65 and 0.50 respectively. According to on-site investigations, the water utilization efficiency in Guanzhong irrigation areas has been improved to 0.55. Thus, if taking ξ_c as 0.65, then ξ_f would reach to 0.85 (Wang et al., 2012b). According to the technical specification for water–saving irrigation engineering (GB/T 50363-2006) (Ministry of Housing and Urban-Rural Development of the P. R. China, 2006), the irrigation water utilization coefficient and the canal conveyance coefficient in large irrigation areas shall be no less than 0.50 and 0.55, and the utilization coefficient of field water in irrigation areas for growing rain-fed crops shall be no less than 0.90 (*Table 1*). Through comparison with the specifications, we can learn that, after implementing water-saving projects in Guanzhong irrigation areas, the utilization coefficient of irrigation water and the canal conveyance coefficient have met the requirements of the specifications, but the utilization coefficient of field water is still at a low level. Therefore, the resource-based water-saving potential is mainly from improving the utilization coefficient of field water, which can be improved by applying land leveling technology, ridged field improving technology, surge flow irrigation technology, and conduit conveyance-based sprinkling and micro-irrigation technologies.

Table 1. *Water utilization coefficient in the specification*

Technical specification for irrigation projects with low pressure pipe conveyance (GB/T 20203-2006) (Standardization Administration of the P. R. China 2006)	**The utilization coefficient of canal water ≥ 0.95**	**The utilization coefficient of field water ≥ 0.85**	**The utilization coefficient of irrigation water ≥ 0.80**
Technical specification for water–saving irrigation engineering (GB/T 50363-2006) (Ministry of Housing and Urban-Rural Development of the P. R. China 2006)	Large-scale irrigation ≥ 0.55 Medium-sized irrigation ≥ 0.65 Small-scale irrigation ≥ 0.75 Canal seepage control ≥ 0.90 Pipeline convey water ≥ 0.95	Rice based irrigation ≥ 0.95 Dry crops based irrigation ≥ 0.90	Large-scale irrigation ≥ 0.50 Medium-sized irrigation ≥ 0.60 Small-scale irrigation ≥ 0.70 Well irrigation ≥ 0.80 Drip irrigation ≥ 0.90

Meanwhile, some of Guanzhong irrigation areas are well-canal combined irrigation areas, in which the regional conduit conveyance coefficient (the percentage of conduit conveyance ≥ 0.95) could be greatly increased through implementing conduit conveyance, thus improving the utilization coefficient of irrigation water in the irrigation areas. If the conduit conveyance coefficient of the conduit-based irrigation areas (such as sprinkling irrigation areas, micro-irrigation areas, and trickle-irrigation areas) is 0.95 and the utilization coefficient of field water is 0.90, then the utilization

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coefficient of irrigation water in the irrigation areas could reach up to 0.855, greatly improving the utilization efficiency of irrigation water. If total water demand stays unchanged, saving of irrigation water could be achieved by increasing conduit conveyance coefficient. Possible resource-based water-saving amount in different situations could be calculated according to crop planting structure and water consumption structure in different Guanzhong irrigation areas.

Resource-based AWP of typical irrigation area

In order to determine the precipitation at the GRP of 25%, 50%, 75%, and 90% respectively, we carried out a precipitation frequency analysis by using the irrigation areas' meteorological data from 1955 to 2015 through Pearson type III curve (Bobée, 1975; Feng et al., 2014), and further calculated the AWPs of the irrigation areas in the corresponding years. *Table 2* shows the resource-based AWP of the Baojixia irrigation area.

Table 2. *The resource-based AWP of Baojixia Irrigation Area in different hydrological years*

Hydrological year (GRP)	Water withdrawal (10^4m^3)	Utilization coefficient of irrigation water		Water-saving amount (10^4m^3)
		Current situations	Using water-saving technologies	
25%	14 893	0.55	0.585	891
50%	33 609	0.55	0.585	2 011
75%	33 609	0.55	0.585	2 011
90%	48 897	0.55	0.585	2 925

*Through the actual investigation, we have learned that under the current conditions of irrigation water use coefficient is 0.55, higher than the target value 0.50 of the transformation of Guanzhong Irrigation Area. After using of water-saving technology, the water use coefficient is raised to 0.585, in which channel water use efficiency is 0.65 with reference to the condition of Guanzhong Irrigation Area reconstruction. According to TSWI (GB/T 50363-2006), the field-water use coefficient is 0.90.

Efficiency-based AWP of typical irrigation area

A covering layer can significantly reduce crops' water consumption. In Guanzhong irrigation areas, covering materials are lacking in the planting season of winter wheat, and covering technologies are seldom used at that time. Therefore, in the growing period of winter wheat, φ_2=0. Large combines are generally used to harvest wheat in Guanzhong irrigation areas. When harvesting, the combines would only take wheat grains away, leaving straw on the soil surface. In addition, wheat-maize continuous cropping system is generally employed in Guanzhong irrigation areas, so summer maize is often covered by wheat straw and stubble during its growing period. According to the studies(Chen et al., 2004; Li et al., 2012), thanks to the covering layer formed by wheat stubble, the evapotranspiration of summer maize would decrease by 10-20% to about 30-50 mm. In addition, straw mulching can increase the water utilization efficiency of fruit trees by 20-30% (Liu et al., 2013) in general, and according to the equation for calculating water utilization efficiency, the water consumption of the trees would decrease by 15-25% with little change in yield. If fruit trees' annual water consumption is 600 mm, then their water consumption would decrease by 90-150 mm after applying covering technologies. In Guanzhong irrigation areas, single cropping system is generally used for planting cotton. Due to soil preparation and other factors, there is

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little possibility that the stubble of the previous crop is used as the covering layer. Therefore, it is more likely to implement plastic film mulching. As indicated by literature, plastic film mulching can decrease the water consumption of cotton by 13-18% in general (15% on average) (Zuo et al., 2010; Liu et al., 2013). The growing period of rape in the Guanzhong Plain is generally from September to May of the next year, and covering technologies are seldom used in this period. When calculating efficiency-based water-saving potentials, conditions of using the water-saving measure of covering to improve crops' water utilization efficiency were mainly considered in this study, with the measure of changing crops' physiological structure being put aside. The percentages (φ_2, namely) of reduced water consumption of different crops in the Guanzhong region, which is achieved by using covering technologies, are shown in *Table 3*.

Table 3. *The Efficiency-based AWP under mulching condition of Baojixia Irrigation Area*

Hydrological year (GRP)	Crop	Winter wheat	Summer corn	Cotton	Fruit tree	Cole
	φ2	**0**	**15%**	**15%**	**20%**	**0%**
	Area of mulching (50% of the acreage) (hm^2)	**51780**	**45353**	**180**	**27200**	**4467**
25%	Evapotranspiration (mm)	407	374	554	634	345
	Precipitation (mm)	161	540	654	654	343
	Evapotranspiration under mulching condition (mm)	407	318	471	507	345
	Theoretical AWP ($10^4 m^3$)	0	1697	10	2300	0
	Actual AWP ($10^4 m^3$)	0	0	0	0	0
50%	Evapotranspiration (mm)	407	364	554	591	345
	Precipitation (mm)	162	340	452	452	243
	Evapotranspiration under mulching condition (mm)	407	309	471	473	345
	Theoretical AWP ($10^4 m^3$)	0	1653	10	2 145	0
	Actual AWP ($10^4 m^3$)	0	0	10	2 145	0
75%	Evapotranspiration (mm)	404	376	554	598	345
	Precipitation (mm)	136	326	430	430	211
	Evapotranspiration under mulching condition (mm)	404	320	471	478	345
	Theoretical AWP ($10^4 m^3$)	0	1707	10	2 169	0
	Actual AWP ($10^4 m^3$)	0	0	10	2 169	0
90%	Evapotranspiration (mm)	407	383	554	609	345
	Precipitation (mm)	149	199	340	340	172
	Evapotranspiration under mulching condition (mm)	407	326	471	487	345
	Theoretical AWP ($10^4 m^3$)	0	1 739	10	2 209	0
	Actual AWP ($10^4 m^3$)	0	1 739	10	2 209	0

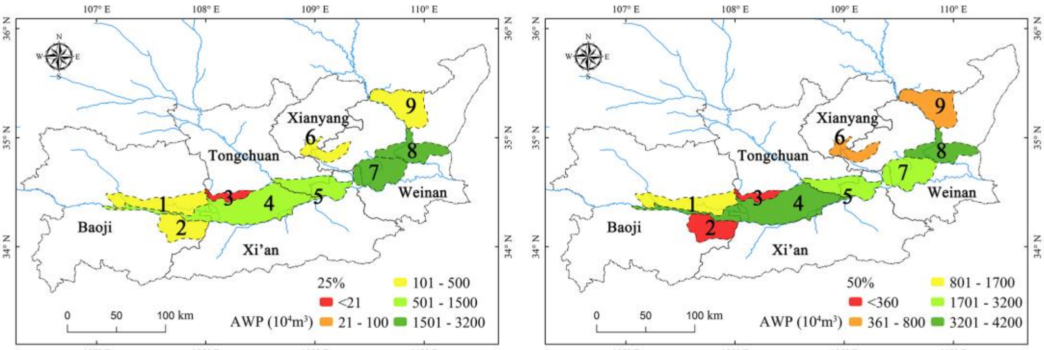
On the premise that the yield does not decrease, the reducible irrigation water amount, which is achieved by applying covering technologies, can be regarded as the saved water amount. According to the irrigation habits in the Guanzhong region, winter irrigation, spring irrigation, and summer irrigation would be generally carried out in a year to meet crops' water demands in different seasons. Therefore, it is necessary to fully consider crops' water deficit and local irrigation habits when making irrigation plans for different hydrological years and calculating corresponding water-saving amount. The irrigation systems for crops in the Baojixia irrigation area for different hydrological years and savable irrigation water amount are described in *Table 4*.

Table 4. *The AWP and irrigation schedules of Baojixia Irrigation Area in different hydrological years*

Hydrological year (GRP)	AWP ($10^4 m^3$)			Irrigation schedules (irrigation amount/ irrigation times) (mm/dimensionless)				
	Total	Resource-based	Efficiency-based	Winter wheat	Summer corn	Cotton	Fruit tree	Cole
25%	891	891	0	247/2	0/0	0/0	0/0	0/0
50%	4 165	2 011	2 154	195/2	0/0	0/0	0/0	0/0
75%	4 190	2 011	2 179	268/2	0/0	0/0	0/0	134/1
90%	6 883	2 925	3 958	258/2	126/1	147/1	131/1	173/2

AWP of each irrigation area

The *Fig. 2* shows the distribution of water-saving potentials of the nine major Guanzhong irrigation areas. It can be seen from total water-saving potentials and unit-area water-saving potentials of all the irrigation areas that total water-saving potentials and unit-area water-saving potentials varied greatly among the irrigation areas. The factors influencing total water-saving potentials include area of irrigation areas, and planting proportion of main crops. It can be seen from *Fig. 2* that the relatively large Baojixia irrigation area had relatively high total water-saving potential, while the relatively small Yangmaowan irrigation area and Taoqupo reservoir irrigation area had relatively low total water-saving potentials. The Jinghuiqu irrigation area, Jiaokouchouwei irrigation area, Luohuiqu irrigation area, and Shibaochuan irrigation area are close in size, but the former three irrigation areas had larger total water-saving potentials than the Shibaochuan irrigation area; that's because the planting area of summer maize and fruit trees in the former three irrigation areas was significantly higher than that in the Shibaochuan irrigation area. *Fig. 3* shows the proportions of resource-based water-saving potentials and efficiency-based water-saving potentials, and it can be seen that the proportions of efficiency-based water-saving potentials of all the irrigation areas were more than 50%.



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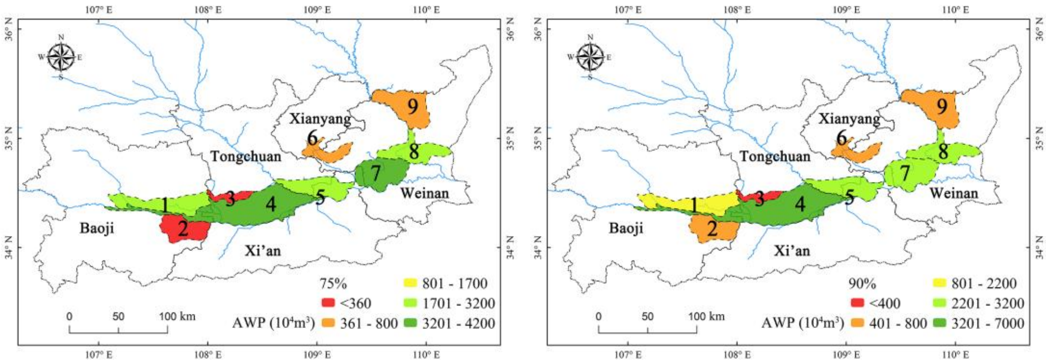

Figure 2. *Map of the total AWP of each irrigations under different GRPs*

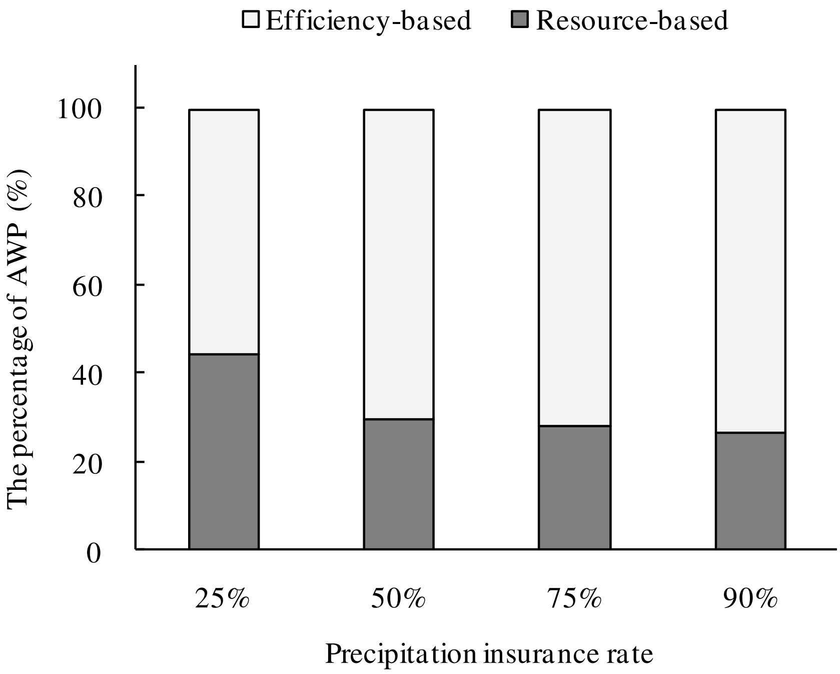

Figure 3. *The proportion of the resource-based (efficiency-based) AWP in Guanzhong Irrigation Area under different GRPs*

AWP in extreme drought condition

In extreme weather conditions, surface evaporation and plant transpiration could be reduced by using covering technologies, and thus irrigation water would be reduced and irrigation water resources would be saved. In the Guanzhong Plain, covering technologies are generally not used in planting wheat; in the growing period of maize, wheat straw is generally used to cover the ground; wheat straw or plastic film mulching could be used in planting fruit trees; plastic film mulching is generally used in planting cotton; covering technologies are generally not used in planting rape. According to relevant data (Wang et al., 2012b), when applying covering technologies, the water-saving percentages (φ_2) of summer maize, fruit trees, and cotton are respectively 15%, 20%, and 15%. Based on the percentages, water-saving amounts of different crops were

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calculated. Water-saving potentials of main crops in Guanzhong irrigation areas under extreme drought condition are shown in *Fig. 4* and *Fig. 5*.

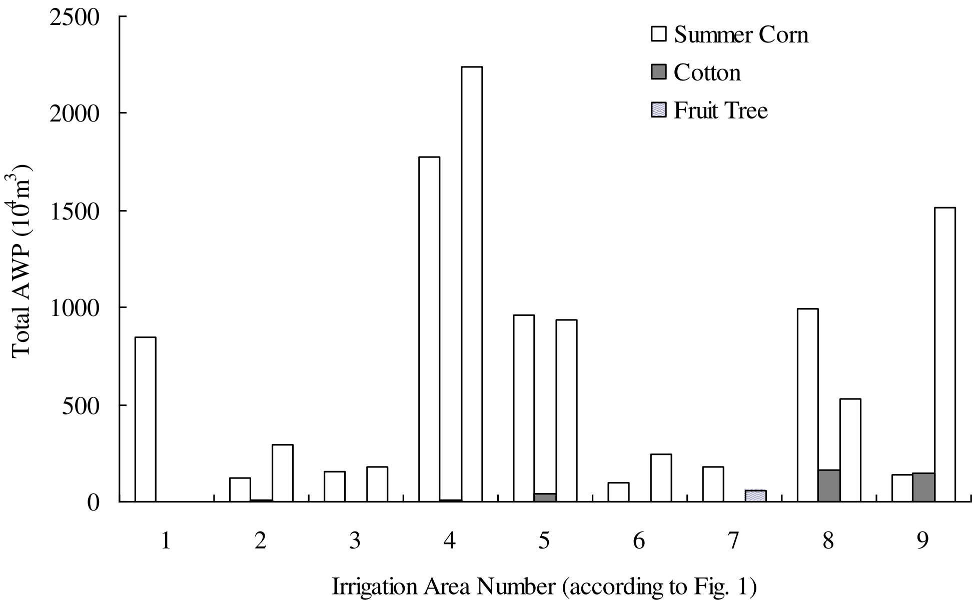

Figure 4. *The total AWP of main crops in Guanzhong irrigation areas in extreme drought condition*

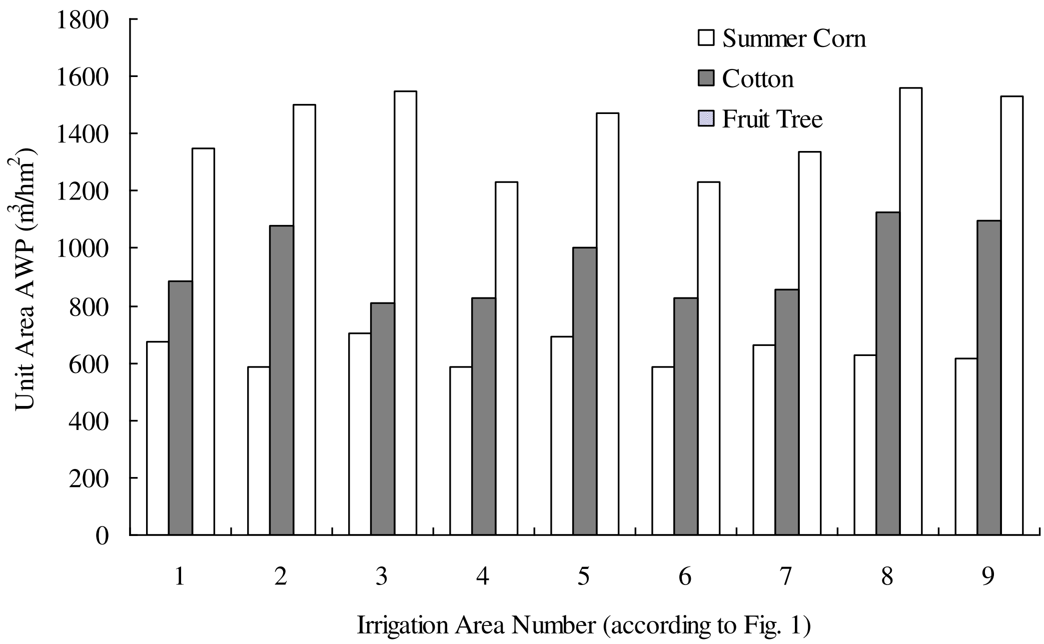

Figure 5. *The unit area AWP of main crops in Guanzhong irrigation areas in extreme drought condition*

From *Fig. 4* and *Fig. 5*, it can be seen that the total water-saving potential of each irrigation area was mainly contributed by summer maize and fruit trees, which accounted for more than 95% of the total water-saving potential. By contrast, winter

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wheat and rape had very small water-saving potentials, which are not shown in the figures. In terms of unit-area water-saving amount, fruit trees, cotton, and summer maize had highest water-saving efficiency, much higher than that of rape and winter wheat. The unit-area water-saving amounts of fruit trees, cotton, and summer maize did not vary much among the nine major irrigation areas, with their average values being 9.4, 6.3, and 4.3 mm respectively. This result is consistent with the actual situation that the irrigation areas are located in the same climate zone and were at a similar technical level of agricultural production.

By using the main crops' water-saving potentials, the total water-saving potentials and unit-area water-saving potentials of the Guanzhong irrigation areas were obtained, as shown in *Figs. 6-8*.

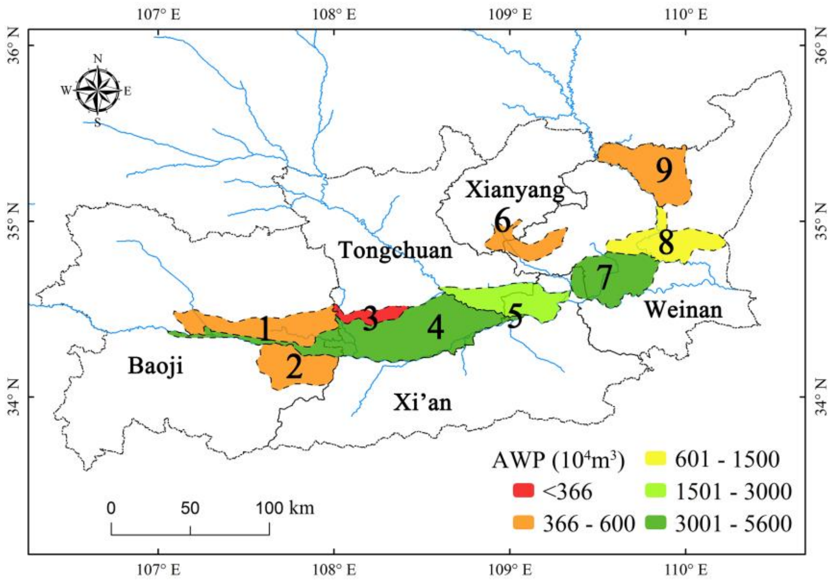

***Figure 6.** The total AWP in each irrigation area in extreme drought condition*

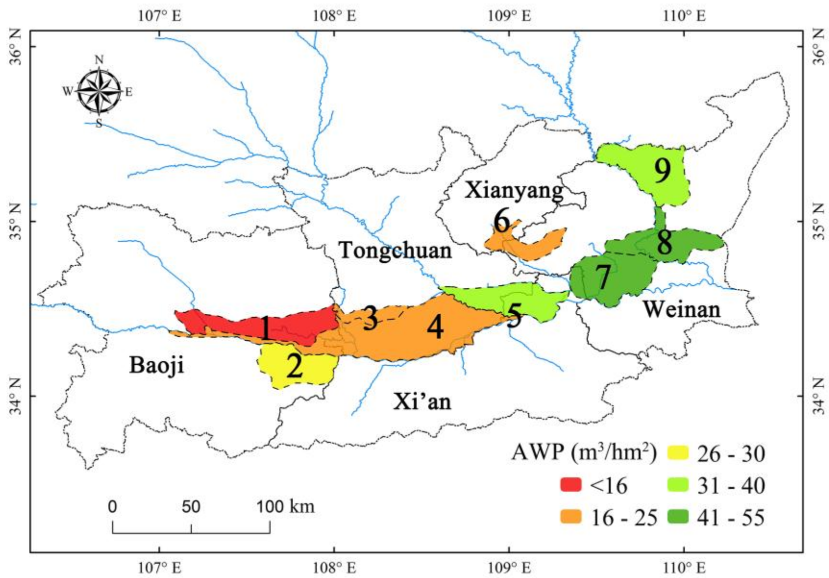

***Figure 7.** The unit area AWP in each irrigation area in extreme drought condition*

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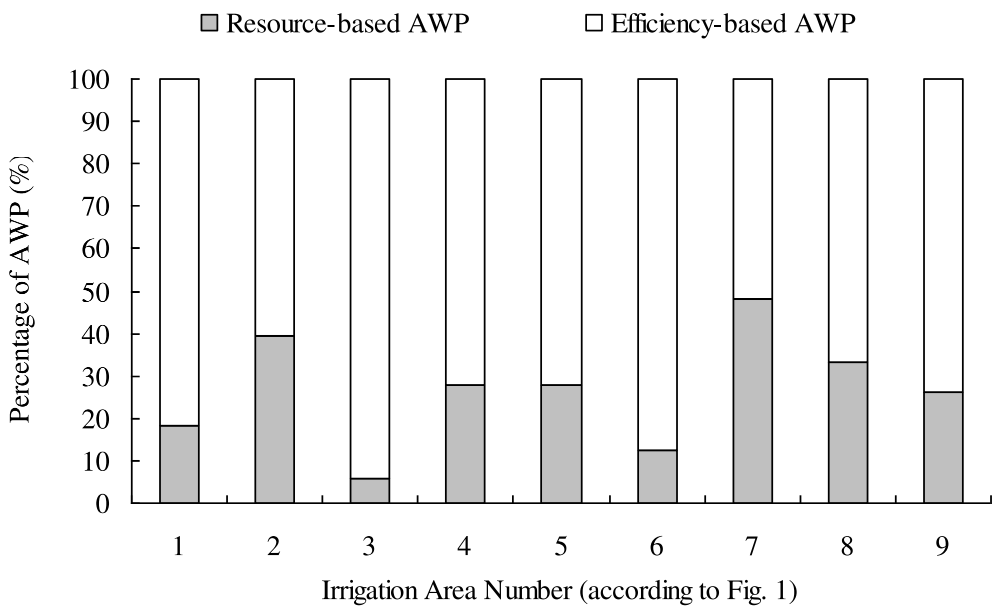

Figure 8. *The percentage of the resource-based (efficiency-based) AWP in each irrigation area in extreme drought condition*

It can be seen from total water-saving potentials and unit-area water-saving potentials of all the irrigation areas in extreme climate conditions that total water-saving potentials and unit-area water-saving potentials varied greatly among the irrigation areas. The factors influencing total water-saving potentials include area of irrigation areas, and planting proportion of main crops. It can be seen from *Fig. 6* that the relatively large Baojixia irrigation area had relatively high total water-saving potential, while the relatively small Yangmaowan irrigation area and Taoqupo reservoir irrigation area had relatively low total water-saving potentials. The Jinghuiqu irrigation area, Jiaokouchouwei irrigation area, Luohuiqu irrigation area, and Shibaochuan irrigation area are close in size, but the former three irrigation areas had larger total water-saving potentials than the Shibaochuan irrigation area; that's because the planting area of summer maize and fruit trees in the former three irrigation areas was significantly higher than that in the Shibaochuan irrigation area.

At different hydrologic conditions (GRP of 25%, 50%, 75%, 90%, and 95%), the proportions of resource-based water-saving potentials and efficiency-based water-saving potentials show that the proportions of efficiency-based water-saving potentials of all the irrigation areas were more than 50%. Thus it can be concluded that improvement of crops' water utilization efficiency should be in preference to improvement of water conveyance efficiency in technology innovation.

Discussion and Conclusions

Concepts and connotations of resource-based water saving and efficiency-based water saving were proposed in this study, and models for calculating water-saving potentials of the two types were established. Key parameters of the calculation models were studied in depth, and the resource-based water-saving coefficient and efficiency-based water-saving coefficient of the main crops in the typical irrigation area (Baojixia)

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were determined based on water-saving schemes for different crops. Based on the established models for calculating water-saving potentials, water-saving potentials of different crops in different water-saving schemes were calculated. In addition, possible resource-based water-saving potentials and efficiency-based water-saving potentials of the nine major irrigation areas in different hydrological years were calculated respectively.

Based on different irrigation areas' planting structure and planting scale, agricultural water-saving schemes were proposed. For the food crops-oriented Shitouhe irrigation area, it is recommended to mainly apply land leveling technology, surge flow irrigation technology or sprinkling irrigation technology, and straw mulching technology. For the commercial crops-oriented Jiaokou and Shibaochuan irrigation areas, it is recommended to apply micro-irrigation technologies and covering technologies. For the cotton-oriented Luohuiqu irrigation area, it is recommended to apply land leveling technology, surge flow irrigation technology, and covering technologies, and to popularize field sprinkling irrigation and trickle irrigation technologies appropriately. For the Baojixia, Fengjiashan, Jinghuiqu, and Yangmaowan irrigation areas, which are oriented at planting food crops and commercial crops, it is recommended to apply land leveling technology, surge flow irrigation technology, and straw mulching technology according to actual circumstances, to popularize field sprinkling irrigation technology appropriately, and to vigorously popularize trickle irrigation technology in areas for planting commercial crops.

Currently, comprehensive definitions of water-saving agriculture and water-saving potential have not been given authoritatively in the research field. As a result, there are numerous directions in researches on specific issues. Thus, in researches on water utilization efficiency, it is still necessary to find out which calculation model is most practical, particularly in calculating water amount. Water-saving potentials vary greatly among research objects. Currently, researches are mainly carried out on improvements to diversion and irrigation projects and agronomic measures in the field, which actually would bring relatively static potentials. In addition, scale effect problem exists in the process of studying water utilization efficiency and water-saving potential. Regarding components of water amount, many scholars have proposed 3 scales in water balance calculation, which are respectively field scale, mesoscale, and macro-scale. As indicated by this study, resource-based water saving is more suitable for micro-scale and mesoscale, while efficiency-based water saving is more suitable for macro-scale. However, effective and reasonable methods to eliminate scale effect and realize conversion between different scales have not yet been proposed and will be an emphasis in future research.

In recent studies, AWP is generally taken as the difference between irrigation water use in a base year and that in a planned year. However, the base year is often selected at will without a standard, and the concept in this sense is not yet perfect due to the scale effect problem. Water-saving irrigation could be applied not only in irrigation agriculture, but also in dryland agriculture. However, applications in the latter are fewer than in the former, so studies on water-saving potential are mainly targeted at irrigation agriculture. In addition, studies on precipitation accumulation and utilization in the region where irrigation agriculture prevails should be enhanced.

The utilization coefficient of irrigation water is an important indicator for evaluation in water-saving agriculture, which is generally taken as the product of the utilization coefficient of channel water and the utilization coefficient of field water. Loss of

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irrigation water would directly influence the utilization efficiency of irrigation water. From this perspective, the factors influencing the utilization coefficient of irrigation water mainly include size of irrigation areas, diversion discharge, groundwater level, soil conditions, and types and technologies of anti-percolation works in irrigation areas. The following problems need to be solved in obtaining the coefficients: heavy workload; it is difficult to guarantee stable measuring conditions (that is because irrigation measurement is generally carried out in production period, during which irrigation water flow, length of channels, existence of branches, and other conditions are subject to changes, thus making measurement very difficult); a great deal of talents possessing special technical knowledge are needed in measuring the coefficients; representative evaluation of the determined coefficients is necessary. The above-mentioned problems need to be discussed in future studies.

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LONG-TERM CONSUMPTION AND FOOD REPLACEMENT OF NEAR-ISOGENIC BY BT-MAIZE ALTER LIFE-HISTORY TRAITS OF *FOLSOMIA CANDIDA* WILLEM 1902 (COLLEMBOLA)

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Abstract. The long-term effect of Bt-maize on soil animals is an important, repeatedly discussed, but a poorly investigated topic. The collembolan *Folsomia candida* is a recognised representative of the mesofauna in the laboratory ecotoxicological experiments. The following questions were addressed in the present experiment: (i) is there any consequence on some life-history traits of *F. candida*, if Bt-maize is provided as food after about 4 years of feeding instead of the near-isogenic counterpart, and (ii) is there any effect on life-history traits, if the near-isogenic and Bt-toxin containing food is changed? Long-term (40-48 generations) feeding on Bt-maize leaf caused alterations in some life-history traits (larger eggs, higher growth rate both in length and width). On the other hand, reproduction (egg size, the total number of eggs, the number of eggs in the first clutch) was influenced, if Bt-toxin containing maize was replaced by its near-isogenic counterpart. Based on currently available methodologies, it is not possible to judge, whether observed differences are due to the Bt-toxin or another component of the maize leaf.
Keywords: *MON810 maize; Cry1Ab toxin; egg size; body growth; sublethal effect*

Introduction

Genetically engineered (GM) crops are cultivated on 179.7 million hectares all over the world from which maize plants are planted on 53.9 million hectares (James, 2015). Side effects of GM plants on soil animals have been studied both in the field as well as laboratory experiments in numerous cases. The results of the field experiments are summarised e.g. by Birch et al. (2007) and currently by Arias-Martín et al. (2016). In general, no severe side-effects on soil animal (Zwahlen et al., 2007; Hönemann et al., 2008) or collembolan (Marvier et al., 2007; Comas et al., 2014) populations and coenosees were found in the field.

Laboratory experiments were performed with GM plants and several non-target soil animal species, like earthworms, woodlouse, nematodes, collembolan, etc. Collembolans occupy a substantial position in plant litter decomposition processes, forming soil microstructure and regulating microbial species composition and biomass (Bakonyi, 1989; Rusek, 1998). That is why collembolan species are used in the laboratory for soil biological studies. The most frequently employed species of them is *Folsomia candida* Willem 1902 (Fountain and Hopkin, 2005). This species have been often in the focus of ecotoxicological studies as well as when the side-effects of the GM plants is examined (Jepson et al., 1994). Besides, this species is recommended as a representative of decomposers, if GM plant and/or Bt-protein effect on non-target organisms is investigated (Römbke et al., 2010; De Schrijver et al., 2016).

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Studying the side-effects of the GM plants on *F. candida*, short-term laboratory studies were conducted in most cases. Tested material included purified toxin as well as different plant parts as pollen, stalk, root or leaf. Their effects were investigated on different life-history traits of *F. candida*, as mortality, reproduction, and growth.

Four Bt-toxins (Cry1Ab, Cry1Ac, Cry2A, Cry3A) presented in the diet (mixed with Baker's yeast) did not influence adult mortality and the number of juveniles o*f F. candida* (Sims and Martin, 1997). Similarly, in a dietary exposure assay, Cry1C and Cry2A toxin did not influence either the survival, and development of the animals, nor the measured antioxidant-, detoxification-, and digestion-related enzyme activities (Yang et al., 2015). However, Broza et al. (2001) found decreased reproduction if *F. candida* was fed on B. thuringiensis diet.

Yu et al. (1997) studied the effect of the Bt-toxin (Cry1Ab and Cry1Ac) producing cotton on oviposition time, the number of eggs, and final body lengths of the *F. candida*, but the measured traits remained unaffected. Romeis et al. (2003) did not find differences in mortality, clutch size, body weight and duration of egg development when *F. candida* was fed on the KP4-transgenic wheat plant (Golin and Greina varieties) and its near-isogenic counterpart. The effects of Bt and near-isogenic lines of rice were studied in two laboratory experiments. In the first one, Bai et al. (2011) used two Cry1Ab containing Bt-rice lines and their near-isogenic counterpart as food. Growth, reproduction, and superoxide dismutase activity were unaffected both in the Petri-dish and soil microcosm experiment. In the second study, root, stem, and leaf of three Bt and non-Bt-rice were provided as food and survival, reproduction and growth as traits were measured (Yuan et al., 2013). Differences in the measured traits between the treatments were not observed in these two studies.

Regarding the effect of the GM maize consumption on *F. candida*, few studies were published till now. Comparing the effects of near-isogenic and Bt-maize (Bt11 and MON810 varieties), no significant differences were detected as a consequence of feeding on Bt-maize in mortality and offspring number by Clark and Coats (2006). Bakonyi et al. (2006) observed about 30% less fecal pellets around the Bt-maize (MON810) than its near-isogenic counterpart. Recently, Zhang et al. (2017) found no effect of Cry1Ab/Cry2Aj toxin contained maize pollen on *F. candida* survival, development, and reproduction.

As presented above, several short-term experiments with *F. candida* exist, but long-term laboratory studies are scarce. As far as we know, no other research group as ours is working in this research field. In a previous study (Bakonyi et al., 2011) no effect of Bt-maize (MON810) on egg production and food choice of *F. candida* was found if the animals were fed up to 29 months with this plant leaves. Working under similar experimental circumstances as in the mentioned study, the following questions were addressed in the present study: (i) is there any consequence on life-history traits of *F. candida*, if Bt maize is provided as food after about 4 years (40-48 generations) of feeding instead of the near-isogenic counterpart, and (ii) is there any effect on life-history traits, if the food is changed, e.g. near-isogenic maize line is provided after feeding on Bt-maize for 4 years? It was hypothesized that (i) long-term feeding has an impact on life-history traits, because of the different quality of the Bt and near-isogenic plants and (ii) a shift due to the food change will appear in life-history traits of *F. candida*, because this species preferred near-isogenic maize leaves in a previous experiment (Bakonyi et al., 2006).

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Material and Methods

Folsomia candida Willem 1902 (Collembola, Isotomidae) used in this study was obtained from the stock population reared in the laboratory of the Szent István University, Department of Zoology and Animal Ecology, Gödöllő (Hungary) since about 20 years. Our animals are belonging to the clade B according to the grouping by Tully et al. (Tully et al., 2006). Collembolans were kept in Petri dishes of a diameter 9 cm based on the method of Goto (1960). Animals in stock culture were kept at a temperature of 20±0.2 °C, with constant humidity (~ 100%) and in total darkness. Dry baker's yeast was given as food ad libitum once a week.

This experiment was started with two stock populations. One of them was fed with MON810 (YieldGard®) maize line and the second one with its near-isogenic counterpart (variety DK-440). Both groups of collembolans were fed with ground leaf of these maize varieties for 4 years. Maize leaves of similar size and position on the plant were collected at harvest than stored dried, in a dark, cool place. Since the embryonic development of our animals takes about 8-10 days, and they arrive their sexual maturity in 10-12 days, its generation time is calculated as roughly one month. Consequently, 10-12 generation per year as a conservative estimation was used for calculating the total number of generations under conditions of our laboratory.

Ground leaf litter was presented as food ad libitum and two grains of baker's yeast to arrange balanced nutrition of the animals. Nitrogen and carbon content of the maize leaf was as follows: N% 0.27 ± 0.02 and 0.29 ± 0.03 and C% 41.9 ± 4.2 and 40.5 ± 4.5 for isogenic and Bt-maize, respectively. Cry1Ab concentration was 8.38 ± 0.19 µg/g dry leaf material, which is in the range presented by Székács et al. (2010). Leaf of MON810 used in this study contained higher Cry1Ab concentration, in comparison with root, stem, anther wall and pollen (Székács et al., 2010).

The experiment was performed with 10-12 days old synchronised *F. candida* individuals. Every treatment of the collembolans was started with 50 animals. They were kept in 5 cm high plastic dishes (the base 5.3 cm and the top 6.6 cm in diameter). Two cm of the mixture of active carbon and plaster of Paris was layered on the bottom in a ratio of 1:10. The top of the wet mixture was scrubbed to arrange smooth surface without holes. The animals were kept individually. Each collembolan got a unique identification number. The experimental animals were kept in total darkness, at a temperature of 20±0.2 °C and with constant humidity (~ 100%). The length of the experiment was 29 days.

Four treatments were set up in the experiment, as follows:

- IsoIso treatment: collembolans consumed the near-isogenic maize (DK-440) for 4 years before the experiment and during the experiment, as well.
- IsoBt treatment: collembolans consumed the near-isogenic maize (DK-440) for 4 years, but MON810 maize during the experiment.
- BtBt treatment: collembolans consumed MON810 maize for 4 years before the experiment and during the experiment, as well.
- BtIso treatment: consumed MON810 maize for 4 years, but near-isogenic maize (DK-440) during the experiment.

Following traits were measured in this study: 1.) final length (mm), 2.) length growth rate, 3.) final width (mm), 4. width growth rate, 5.) number of eggs in the first clutch, 6.) days till first clutch, 7.) total No. of eggs, 8.) No. of eggs/clutch, 9.) egg size (mm).

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Digital photos were taken from each animal twice a week from 4 to 29 days (Olympus C7070 Wide zoom camera with Olympus C5060 ADL optic). The length of the animals was measured from the frons to the end of the last segment, while the width at the widest part of the metathorax. The length and width for any collembolan at a given time were calculated as the mean of measurements on two consecutive photos to avoid inaccuracy which may cause of collembolan movements. Final body length and width, as well as growth rate, were calculated with the CurveExpert 1.4 software for any animals (Hyams, 2010). The equation Exponential Association (y = a(1-e^-bx; where a = final value of length or with; b = growth rate) was applied. Only those animal's data were regarded which survived until the end of the experiment.

The egg clutches were transferred to Petri dishes which contained a 0.5 cm high mixture of active carbon and plaster of Paris (1:10) on the bottom. The clutches were spread carefully with a wet brush in order to separate eggs from each other and digital photo was taken from them (BTC STM-9T microscope equipped with MicroQ-PRO type camera). The eggs were numbered on the photo. After that, an egg was chosen randomly from all of the clutches. The shortest and longest diameters of the eggs relative at a 90° angle to each other were measured with the aid of the ImageJ software (Schneider et al., 2012). Egg diameters were transformed so that the two diameters were multiplied by each other and the square root of it was extracted, so it gives a diameter as the egg were perfectly round (TD = egg size, hereafter). Egg numbers and the date of egg laying were monitored continuously every day during the experiment.

Final length, length growth rate, final width and width growth rate were compared with ANOVA. If the significant effect was found ($p < 0.05$) than posthoc Bonferroni-Holm test was applied. Effect of treatment on a total number of eggs, the number of eggs in the first clutch, days till the first clutch, the number of eggs/clutch and egg size (TD) was analysed with linear models. The mortality of the animals was analysed with a binomial model (R Core Team, 2012).

Canonical Variate Analysis (CVA) was performed based on the measured traits (the trait "days till the first clutch" was not applied because data distribution was not normal despite transformation). The SynTax2000 statistical program package (Podani, 1993) was used for the analysis.

Results

Long-term feeding effect

At the start of the experiment the body length and width of the 10-12 day old collembolan were significantly different between the treatments ($t = 3.9$, $df = 46$, $p < 0.001$ and $t = 2.8$, $df = 46$ $p < 0.01$, for body length and width, respectively). Growth curves of both length (*Figure 1*) and width (*Figure 2*) showed sigmoid shape type. Measured points fitted well to the curve because correlation coefficients of the fitting were 0.95 or higher in cases of all individuals. Final body length and width of the IsoIso and BtBt animals did not differ significantly from each other (*Table 1*). The length and width of the collembolans ranged from 0.426 to 2.238 mm and 0.080 to 0.583, respectively (data of all treatments). The relationship between body length and width may describe with the equation as follows: width (mm) = 0.27*length (mm) – 0.047 ($r = 0.95$, $t = 88.4$, $df = 1161$, $p < 0.001$). No treatment-related differences in body length and width relationship were found. Growth rates correlated negatively with both final body length ($r = 0.73$, $t = 10.6$, $df = 99$, $p < 0.001$) and width ($r = 0.71$, $t = 10.0$, $df = 99$,

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p < 0.001). This relationship is seen very clearly in *Figure 3*. as well. Egg size and a total number of eggs did not correlate each other.

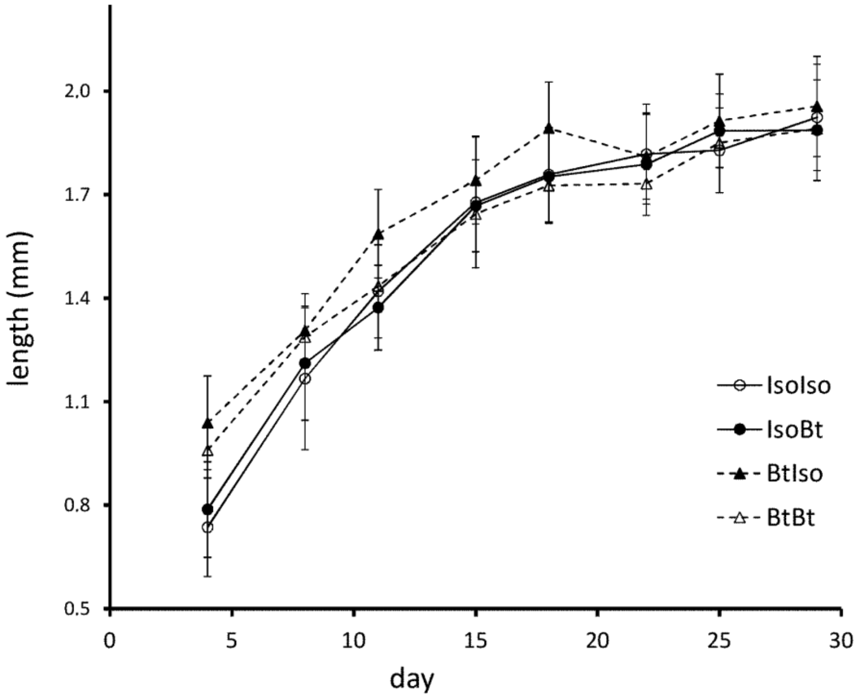

Figure 1. *Body length growth (average±SD) of F. candida in the four treatments during the experiment.*

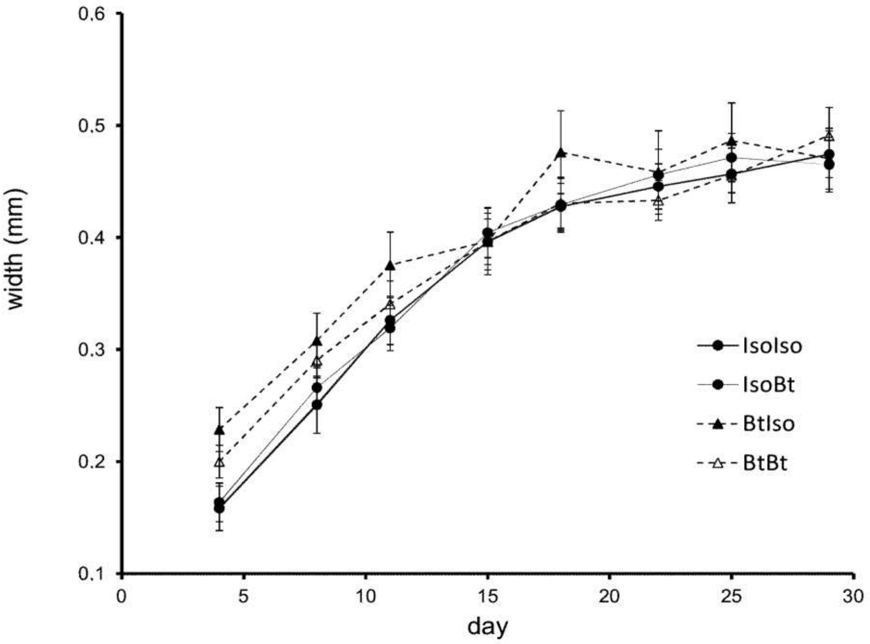

Figure 2. *Body width growth (average±SD) of F. candida in the four treatments during the experiment.*

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Table 1. *Average (±SD) of the measured traits. Means in a row followed by the same letter are not significantly different (p < 0.05).*

	IsoIso	IsoBt	BtBt	BtIso
Final length (mm)	1.99±0.18	1.97±0.16	1.92±0.19	1.95±0.12
Length growth rate	0.13±0.04[a]	0.12±0.02[a]	0.16±0.05[b]	0.17±0.05[b]
Final width (mm)	0.53±0.05	0.54±0.07	0.51±0.09	0.5±0.05
Width growth rate	0.1±0.03[a]	0.09±0.03[a]	0.12±0.04[b]	0.14±.04[b]
No. eggs in first clutch	52.7±41.4	31.0±24.0	33.9±28.6	28.9±24.1
Days till the first clutch	15.3±5.0	14.1±3.3	13.6±4.8	13.8±5.3
Total No. of eggs	131.0±53.1	111.8±41.2	144.5±93.4	120.2±54.8
No. of eggs/clutch	55.8±17.9	45.3±13.0	51.3±23.1	59.8±26.2
Egg size (TD) (mm)	0.100±0.02[a]	0.105±0.03[a]	0.125±0.03[b]	0.110±0.04[b]

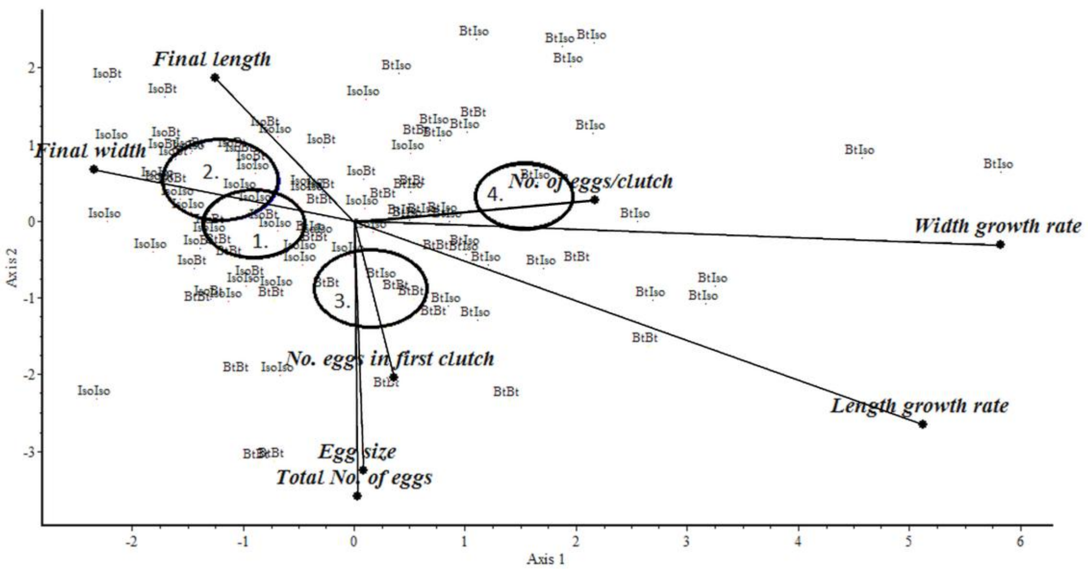

Figure 3. *Results of the CVA analysis. Confidence circles are shown. Numbers of treatments are as follows: 1. IsoIso, 2. IsoBt, 3. BtBt, 4 BtIso.*

The effect of the long-term feeding is demonstrable by comparing data from IsoIso and BtBt treatments. Three out of nine measured traits showed significant differences between these treatments (*Table 1*). Both of the length and with growth rate was higher in the case of the BtBt treatment during the investigated period. Besides, collembolans in BtBt treatment laid significantly larger eggs. No other traits differed between the two treatments (*Table 1*). The mortality of the four treatments was 22, 29, 20 and 28 percent

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in IsoIso, IsoBt, Bt Iso, and BtBt treatment, respectively. No significant differences were detected between these values (p= 0.231, z= -1.197).

Food change effect

Changing of food source did not cause significant alteration in any traits measured in the study (*Table 1*). Neither the food change from Iso to Bt nor vice versa has any statistical difference in life-history traits. However, egg size and total number of eggs correlate negatively each other ($r = 0.41$, $t = 2.4$, $p < 0.03$ and $r = 0.32$, $t = 2.1$, $p < 0.05$ for IsoBt and BtIso treatments, respectively).

Results of the CVA analysis show differences between population traits (*Figure 3*). Eigenvalues as a percent of Axis1 and 2 are 78.7 and 16.1, respectively, i.e. the first two axes explain 94.8 percent of the total variance. Both Axis1 and 2 contribute significantly to separation of the groups (chi2 = 107.1, df = 24, $p < 0.001$ and chi2 = 29.6, df: 14, $p < 0.01$ for Axis1 and 2, respectively). Axis1 determines first of all the growth and Axis2 the reproduction of the *F. candida*. The main factors responsible for the separation of all the four groups are final length and width as well as their growth rates. This result coincides with a negative correlation of final size and growth rate of animals, as it was presented before. The traits, "total number of eggs", and "egg size" distinguish between groups, where food change occurred, especially in the case of the BtIso treatment (*Figure 3*).

The mortality of the four treatments was 22, 29, 20 and 28 percent in IsoIso, IsoBt, Bt Iso, and BtBt treatment, respectively. The alteration of the food did not cause a significant difference in mortality (p= 0.1109, z= -1.594), but the interaction of the long-term feeding and the food change was significantly associated with mortality (p= 0.0345, z= 2.114).

Discussion

According to Zwahlen et al. (2003), the Cry1Ab toxin can persist in the soil up to 2 years. In spite of this fact, a long-term investigation of Cry1Ab toxin producing maize on soil fauna is very rare. That is why Heckmann et al. (2006) and Székács and Darvas (2013) emphasise the significance this kind of studies.

In our previous investigation with *F. candida* (Bakonyi et al., 2011), we did not find a relationship between elapsed time from the start of feeding on the Bt-maize and a total number of eggs produced by one individual. This trait was affected neither in the present, a longer experiment where similar maize lines were used.

It has long been known that food limitation (Martin, 1987), as well as the quality of the food (Boersma and Kreutzer, 2002), can considerably influence life history traits. According to the results of the present study, two slightly different life-history strategies seem to emerge due to long-term feeding on two qualitatively different food sources. Our populations have been fed for 40-48 generations on either near-isogenic or Bt-maize. So many generations may be definitely enough for adaptive changes of life-history and adjust to different food sources (Awmack and Leather, 2002; Fricke and Arnqvist, 2007). Probably, as a consequence of accommodation to food, our collembolans in BtBt treatment produced larger eggs than in IsoIso one. Tully and Ferrière (2008) found that there is a positive relationship between egg size and juvenile size, and larger *F. candida* juveniles have improved quality, which means a higher chance to survive and better productivity. Maybe that is why the time until the first

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clutch is shorter (although not significantly) and the body growth rate is higher in BtBt treatment and BtBt collembolans reach their maturity earlier. In the case of the IsoIso treatment, all these traits are changing oppositely. However, the final body size is equal in both treatments. The result of all these events is the differently shaped growth curves of collembolans in IsoIso and BtBt treatments (*Figure 4*).

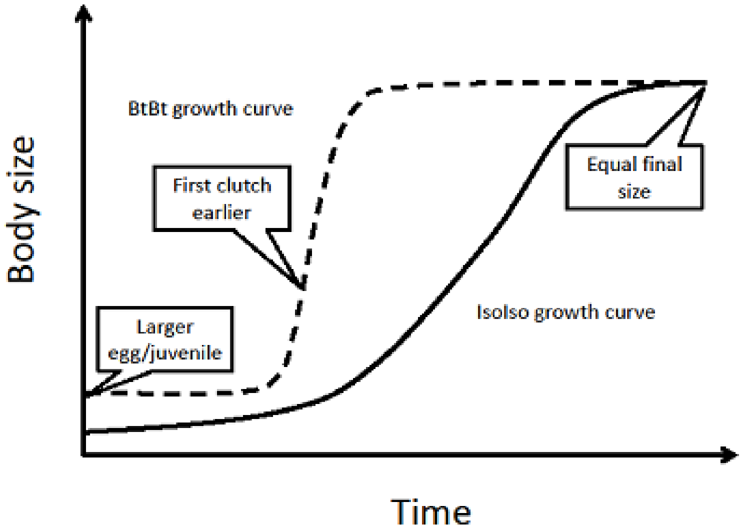

Figure 4. *Schematic presentation of results summary. Differences in body length growth of the IsoIso and BtBt treatments is presented.*

Based on the results of CVA analysis, it was not a noteworthy consequence if the collembolans from the Iso stock breed were fed with Bt-maize during the experiment. Oppositely, considerable modification in reproduction traits was observable, if collembolans from Bt stock breed were fed with near-isogenic leaf during the experiment. Reproduction of the collembolan in the BtIso treatment becomes more similar to IsoIso and IsoBt treatments than to their original BtBt counterparts. Smaller eggs in the BtIso treatment may be a sign of the favorable food (Tully and Ferrière, 2008).

It is well known that food quality has a significant influence on life-history traits of *F. candida* (Jørgensen et al., 2008). So, the contrast between the two life-history strategies observed in this experiment may be the results of the different quality of maize leaves used as food. Nitrogen proved to be one of the main factors, which determine reproduction and growth of *F. candida* in several cases (Booth and Anderson, 1979; Leonard and Anderson, 1991; Kaneda and Kaneko, 2002). However, leaf nitrogen and carbon content were similar in maize leaves in our study. That is why nitrogen was probably not responsible for life-history changes detected in our study.

The higher lignin content of MON810 comparing its near-isogenic counterpart is suggesting as a factor, which can decrease digestibility of this Bt-maize by herbivores (Saxena and Stotzky, 2001). However, they investigated the lignin content of stems and not leaves, and lignin content is different in maize stem and leaf (Johnson et al., 2007). In the case of maize leaf, Tarkalson et al. (2008) did not find differences in lignin content of four maize lines (Pioneer 34N44 Bt, Pioneer 34N43, NC+4990 Bt, NC+4880). Poerschmann et al. (2005) found marginally higher lignin content in the Bt-

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maize leaf than in its near-isogenic counterpart (Novelis, Valmont Bt and Nobilis, Prelude near-isogenic lines). We did not measure the lignin content of the leaves, so the possible lignin effect remained unanswered. Besides, any undetected factor(s), which influenced *F. candida* food choice (Bakonyi et al., 2006), may also be responsible for alteration in reproductive strategy of the BtBt and IsoIso treatments.

Previous studies suggest that qualitative differences between Bt and near-isogenic lines may occur, which explain detected differences of the life-history strategies of the IsoIso and BtBt populations in this study. Our findings are in line with the results of several studies, which revealed life-history adaptation due to food alteration at *F. candida* (Booth and Anderson, 1979; Stam et al., 1996; Tully and Ferrière, 2008).

The exact effect of Cry toxins on the population biology of *F. candida* and other insect species remained an unresolved issue till now. The central problem is that Cry toxins are embedded in the matrix of plant cell cytoplasm, and their effect could be correctly interpreted only in this context. If pure Cry toxins were examined in any experiments, no effect was found (Sims and Martin, 1997; Yu et al., 1997; Galbraith et al., 2015; Yang et al., 2015). It does not mean that possible harm of the toxin could be excluded with confidence because potentiating effects has never been tested yet. However, it remains questioning, whether such a test developable or not. Moreover, if whole Bt-plant or any plant parts was tested in a study, then the current experimental methods did not separate the only effect of any cell component (including Cry toxin) from the other. New methodologies are required in order solving this problem.

Using functional traits to predict responses to environmental changes is in the focus of several contemporary soil ecological studies (Hedde et al., 2012; Salmon et al., 2014). Collembolan functional traits were involved as markers of biodiversity decline (Vandewalle et al., 2010), climate change (Makkonen et al., 2011; Bokhorst et al., 2012), forest fire (Malmström, 2012) and urbanisation (Santorufo et al., 2014). Moreover, Hawes et al. (2009) applied this approach in order to evaluate effects of genetically modified herbicide-tolerant crops on functional groups of soil invertebrates. It was found that this method is suitable for monitoring effects of the new crops introduction in an area. Correspondingly, our result indicates that *F. candida* egg size and growth are appropriate functional traits for such monitoring work.

Conclusions

Long-term (40-48 generation) feeding of *F. candida* on Cry1Ab toxin producing maize (Mon810, YieldGard®) causes considerable differences in this species compared to the treatment group which was fed with near-isogenic maize. Change of life-history parameters causes a difference in the growth curve of animals. On the other hand, a shift in food source (change from Bt to Iso maize) may initiate changes in reproduction. This shift is a quick process, which is observable even in one generation, as it is proved in this experiment.

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THE BIOACCUMULATION AND METABOLIC EFFECTS OF CIPROFLOXACIN-HCL AND CIPROFLOXACIN FREE BASE IN YELLOW LUPIN (*Lupinus luteus* L.) SEEDLINGS

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Abstract. Two forms of ciprofloxacins (CIP) are used in animal husbandry and veterinary medicine, and may occur in manure-fertilized soil as pollutants: CIP-HCl salt, readily dissolvable in water, and the free base CIP, practically water-insoluble. The aim of this study was to compare the phytotoxicity of both forms of CIP to yellow lupin seedlings (*Lupinus luteus* L.). In eight-day-old lupin seedlings higher amounts of both water-soluble and insoluble CIP were detected in roots, compared to shoots. The amount of CIP in plants treated with CIP-HCl was 1.5 times higher compared to plants treated with the insoluble, free base form. Nevertheless, both forms of the antibiotic caused very similar reduction in lupin shoot length, root length and seedling fresh mass as well as very similar increases in seedling dry mass. Activity of guaiacol peroxidase in control seedlings was 14.09 and 49.25 U in roots and shoots, respectively, and in plants treated with CIPs it was highly dependent on the antibiotic dose. Guaiacol peroxidase activity was stimulated in plants treated with 5 mM CIP of any kind (water-soluble or insoluble), and this activation was particularly visible in roots, while the enzyme activity decreased to nearly zero in both roots and shoots of plants treated with CIPs at the level of or above 20 mM. The activity of superoxide dismutase in roots was severely repressed by even the lowest dose of any CIP, while it was slightly stimulated in shoots by any of the CIPs at the level not exceeding 10 mM. The following carbohydrates were detected in roots of lupin grown in soil contaminated with CIPs (water-soluble and insoluble): D-*chiro*-inositol, D-pinitol, *myo*-inositol, galactose, glucose and sucrose. The content of myo-inositol and sucrose in lupin roots increased with increasing concentrations of soluble and insoluble CIPs. Neither of the forms of the drug clearly affected the content of D-*chiro*-inositol, D-pinitol, glucose and galactose. Both forms of CIP showed very similar effects on lupin seedlings, however, the accumulation of CIP-HCl was a little higher compared to the free base form of this antibiotic.
Keywords: *lupin, plant uptake, guaiacol peroxidase and superoxide dismutase activity, soluble carbohydrates*

Introduction

Antibiotics are widely applied in human and veterinary medicine and industrial animal production. The increased usage of well-known forms of these pharmaceuticals, as well as the launch of new antibiotics and the spread of multi-stand industrial farms have resulted in increased environmental pollution with antibiotics and their metabolites. The environmental impact of the majority of these drugs is unknown or is regarded as low. However, their presence may be linked to ecotoxicological changes with negative effects on ecosystems (Ding and He, 2010; Santos et al., 2010).

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Ciprofloxacin (CIP) belongs to the fluoroquinolone group of pharmaceuticals and is one of the most commonly applied antibiotics. It has a broad spectrum of biological activity and is used against both Gram-negative and Gram-positive bacteria. When applied frequently, it infiltrates soil and is detected at the level 6 to 750 µg/kg (Golet et al., 2002; Kemper, 2008; Martínez-Carballo et al., 2007). The mechanism of CIP toxicity towards bacteria involves the inhibition of DNA topoisomerases: topoisomerase II (DNA gyrase) and topoisomerase IV, resulting in an arrest of bacterial DNA replication (Oliphant and Green, 2002). The bioavailability of ciprofloxacin in various mammals is estimated at about 70%. After oral ingestion, about 15% of the drug undergoes transformation into less active metabolites that are detected in urine, while 40-50% is detected as the unchanged form (DrugBank Reports, DB00537).

Drug bioavailability is affected by several factors. These include the properties of the drug itself, as well as the type and quantity of the solvent. Many antibiotics may occur as water soluble salts – hydrochlorides – and water-insoluble, free base forms (Caco et al., 2008). The kinetics of the drug dissolution process affects the drug absorption and accumulation in plants. Plants absorb antibiotics from water and soil and distribute them throughout plant tissues as a result of passive transport in transpiration stream (Liu et al., 2013). Antibiotics in plants may decrease the plant growth rate and activity of biochemical defence reactions (Sikorski at al., 2014). One of the key defence mechanisms in plants is activation of antioxidative enzymes: guaiacol peroxidase (POD, EC 1.11.1.7) and superoxide dismutase (SOD, EC 1.15.1.1). These enzymes are present in nearly all plants and they are important elements of plant antioxidative protection system (Cheng et al., 2012; Passardi et al., 2004; Cesarino et al., 2013). They catalyse the disproportionation of the superoxide O_2^- radical into hydrogen peroxide and molecular oxygen. The reaction occurs in all organisms that metabolise oxygen. Class III peroxidases are secreted glycoproteins that participate in lignin and suberin biosyntheses and deactivate toxic compounds. Moreover, they are involved in the developmental processes by controlling auxin level and cell elongation (Passardi et al., 2004; Bakalovic et al., 2006).

Plant responses to some environmental stresses include the accumulation of soluble carbohydrates, that ensure the right cellular osmotic potential and create a protective layer around macromolecules. They also act as signal substances and take part in antioxidative defence (Noiraud et al., 2001; Rontein et al., 2002; Piotrowicz-Cieślak and Adomas, 2012).

The aim of the study was to compare phytotoxicity of two forms of CIP (water-soluble and insoluble) towards lupin (*Lupinus luteus* L.), cultivar Mister, by assessing selected morphological and biochemical features: seedling growth, the activity of guaiacol peroxidase, superoxide dismutase and the content of soluble carbohydrates as well as quantifying content of both drug forms in lupin roots and shoots.

Material and Methods

Plant material and growth conditions

Seeds of yellow lupin (*Lupinus luteus* L.) cv. Mister were germinated for eight days in Phytotoxkit plates (MicroBio Test, Inc., Belgium). Germination was carried out under controlled climatic conditions with temperature set at 25° C and 90% relative humidity (RH), with a 16h/8h day/night photoperiod and 3.4 klx light intensity. Ninety milliliters of soil (sand, vermiculite, peat 1:0.3:1, v/ v/v) were placed in each plastic

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microbiotest plate. The soil was covered with Whatman No. 1 filter paper and watered with 27 ml distilled water supplemented with water-soluble and insoluble CIP (Sigma-Aldrich) at final concentrations of 5; 10; 20; 40, 80 mM. The control plants were watered with pure distilled water. The root length was estimated after eight days of germination using Image Tool for Windows. Fresh weight of seedlings was determined according to standard seed testing recommendations (ISTA, 1999). The experiment was carried out in four replicates, each containing 40 seedlings.

Water-soluble and insoluble ciprofloxacin content in seedlings

CIP was extracted from eight days-old seedlings grown in soil containing CIP-HCl or CIP free base. Plants were first rinsed with distilled water, then manual solid phase extraction (SPE) was carried out by squeezing the sap from plant material with a mortar and pestle and transferring the sap to SPE cartridges (Chromabond®Easy, 3 ml×200 mg^{-1}, Macherey-Nagel, Dtiren, Germany) prewashed with methanol (Rydzyński et al., 2017). After loading the samples to cartridges the antibiotic was eluted with 250 µl methanol. CIP in seedlings was analyzed by HPLC according to Pailler et al. (2009) with small modifications. Briefly, the chromatographic system consisted of a Water Aliance 2695 HPLC system (Waters Corp.) with a binary high-pressure gradient pump, an automatic injector and a column oven. The chromatographic column was an Atlantis T3 column (150×3.0 mm, 3 µm) (Waters Corp.) at 40^oC. The MS–MS analyser consisted of Quattro micro® API MS (Waters Corp.) using electrospray in the positive mode (ESI+). N_2 was used as nebulizer, drying, curtain and collision gas. A chromatographic gradient was applied for the separation of the analytes depending on the ionization mode employed, with a total chromatographic run of 18 min. Gradient elution was carried out with aqueous 0.1% formic acid : 0.1% formic acid in acetonitrile at a flow rate of 4.5 $ml{\times}min^{-1}$. Validation of the method included the assessment of selectivity, linearity (1 to 11 $\mu g{\times}ml^{-1}$), limits of detection (8 $ng{\times}ml^{-1}$) and quantification (26 $ng{\times}ml^{-1}$). Chromatographic system and data collection were controlled with a MassLynx 4.1. chromatographic software interfaced to a personal computer.

Guaiacol peroxidase activity

Extracts used to determine guaiacol peroxidase (POD) activity were prepared on ice. The root and shoot tissues (500 mg) that grew in soil with water or with chlortetracycline were homogenized for 30 minutes in the extraction buffer (0.1 M Tris/HCl, pH 7.8, 8.75% polyvinyl pyrrolidone, 0.1 M KCl, 0.28% Triton X-100). Samples were centrifuged for 30 minutes at 4000×g at 4ºC. The supernatant was passed through membrane filters with 0.45 µm porosity. The protein content in samples was determined with the Lowry et al. (1951) protein assay. POD was determined based on the spectrophotometric detection (Cecil, CE2021 2000 Series) in a mixture containing 100 µl 1% guaiacol, 2 ml 0.1 M KH_2PO_4, 150 µl supernatant and 20 µl 0.18% H_2O_2. The absorption rate increase was measured at room temperature at the wavelength of 470 nm. One unit of activity equals oxidation of 1 µmole H_2O_2/mg protein during 1 minute. POD analysis was carried out in four replications.

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Superoxide dismutase activity

Extracts used for superoxide dismutase (SOD) activity determination were prepared on ice. The root and shoot tissues (200 mg) were homogenized in the extraction buffer (1ml) consisting of 50 mM phosphate buffer (pH 7.0), 1% polyvinyl pyrrolidone, 0.1 M KCl, 0.1% Triton X-100. Samples were centrifuged for 15 minutes at 15 000×g at 4ºC, and supernatants were used as crude extracts for soluble protein quantification according to Lowry et al. (1951) with bovine serum albumin as the standard. Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (2.2 ml) contained, 0.05 M buffer $Na_2CO_3/NaHCO_3$ (pH 10.2), 0.0001 M EDTA, 0.0001 M xantin, 2.5×10^{-5} M NBT and 50 µl of plant extract. The reduction of NBT by superoxide radicals to blue coloured formazan was followed at 560 nm. One Unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions per 1 g protein.

Soluble carbohydrates contents

Soluble carbohydrates (sucrose, glucose, galactose, *myo*-inositol and D-*chiro*-inositol) content in the seedlings were analyzed by GC chromatography according to Piotrowicz-Cieślak (2005). Tissues (100 mg fresh mass) were homogenized in ethanol : water mix, 1:1 (v/v) containing 300 µg phenyl-α-D-glucose as internal standard. The homogenate and the wash were combined in a 1.5 ml microfuge tube, heated to 75 ºC for 30 min in order to inactivate endogenous enzymes and centrifuged at 15 000×g for 20 min. The supernatant was passed through a 10 000 MW cut-off filter (Lida, Kenosha, WI USA). Aliquots of 0.3 ml filtrate were transferred to silylation vials and evaporated to dryness under a stream of nitrogen. Dry residues were derived from 300 µl of silylation mixture (trimethylsilylimidazole : pyridine, 1:1, v/v) in silylation vials (Thermo Scientific) at 70ºC for 30 min, and then cooled at room temperature. One µl soluble carbohydrate extract was injected into a split-mode injector of a Thermo Scientific gas chromatograph equipped with flame ionisation detector. Soluble carbohydrates were analyzed on a DB-1 capillary column (15 m length, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific) and identified with internal standards as available. Concentrations were calculated from the ratios of peak area, for each analyzed soluble carbohydrates, to the peak area of respective internal standard. Quantities of soluble carbohydrates were expressed as mean ± SD for four replications of each treatment.

Statistical analysis

The experiment was conducted in four replicates. The results were statistically evaluated using Statistica 6.0 software by performing analysis of variance (F test) for two factor experiments (split-plot) at the significance level $p = 0.01$. The mean values of the plots were compared using q SNK test (Student-Newman-Keuls).

Result

The lupin root length was found to decrease due to the presence of the lowest of the tested CIP concentrations, i.e. 5 mM for both drug forms (*Figs 1A, 1B*). CIP at the highest concentration (80 mM), both as the soluble and insoluble form, significantly

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inhibited the growth of roots, by 85% and 75%, respectively (*Figs 1A, 1B*). A similar reaction to the drug was observed for lupin shoots. Shoots of lupin growing in soil contaminated with 5 mM of the water-soluble form of the drug were shorter by 7 mm on average than the shoots of plants growing in soil with the insoluble CIP form (*Figs 1A, 1B*). Fresh mass of roots growing in soil contaminated with 5 mM of water-soluble and insoluble CIP was lower by 35 mg, compared with the control sample. On the other hand, the weight of fresh roots of plants growing in soil contaminated with soluble and insoluble CIP at the highest of the concentrations was lower by 116 mg and 97 mg, respectively (*Figs 1C, 1D*). Fresh mass of lupin shoots also decreased, regardless of the drug form.

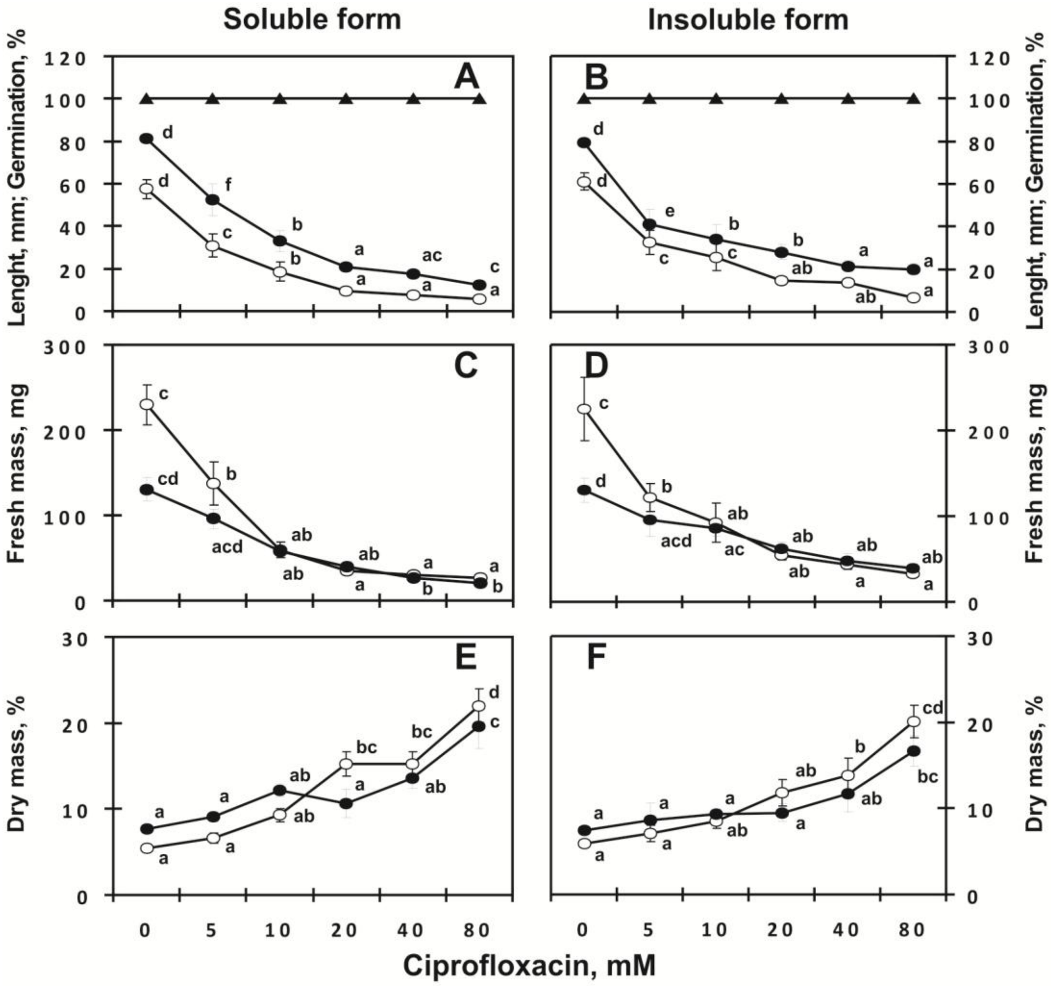

Figure 1. *Panel A and B seedlings length (○ - root, ● - shoot), germination (▲); panel C and D fresh mass (○ - root, ● - shoot); panel E and F dry mass (○ - root, ● - shoot) in yellow lupin after three days on soil supplemented with different soluble and insoluble form of ciprofloxacin concentration (0, 5, 10, 20, 40, 80 mM). Data points represent the means ± SD. Means with the same letter are not significantly different from each other (p≤0.01).*

Control shoots reached a fresh mass of 230 mg, whereas those from plants growing in soil contaminated with the drug water-soluble and insoluble forms at the highest concentrations reached only 27 and 32 mg (*Figs 1C, 1D*). The dry mass of lupin roots

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increased with increasing concentrations of both forms of CIP in the soil. The dry mass of roots in the control sample was 7.6%, whereas it was 19.6% and 16.6%, (*Figs 1E, 1F*), in the case of roots of plants growing in soil contaminated with 80 µM of water-soluble and insoluble CIP, respectively. Similarly, the dry mass of shoots increased steadily with increasing contamination of soil with both forms of CIP. With the highest of the concentrations under study, it reached 22% (water-soluble CIP) and 20% (insoluble CIP) (*Figs 1E, 1F*).

CIP was found to be present in roots and shoots of seedlings treated with both CIP-HCl and CIP free base (*Fig. 2*). The roots, in comparison to shoots, accumulated more CIP in both plants treated with the water-soluble and insoluble CIP form. The contents of the drug in roots of plants grown in soil with 5 µM CIP (both CIP-HCl and CIP free base) were similar and averaged 200 $\mu g \times g^{-1}$ fresh mass. Roots grown in soil contaminated with 80 µM drug contained the highest amount of water-soluble CIP (700 $\mu g \times g^{-1}$ fresh mass) and insoluble CIP (400 $\mu g \times g^{-1}$ fresh mass). Compared to shoots, roots contained 1.5 less of both drug forms. Similar to root tissues, the contents of both drug forms in shoots depended on the level of CIP in soil. The maximum level of CIP in shoots was 380 and 180 $\mu g \times g^{-1}$ fresh mass, for soluble and insoluble form, respectively (*Fig. 2*). Lupin roots took up more of the drug than the shoots. The uptake of insoluble CIP from soil by yellow lupin roots and shoots was slower compared to the soluble form. The drug concentration in the lupin organs increased with increasing contamination of the soil with ciprofloxacin (*Fig. 2*).

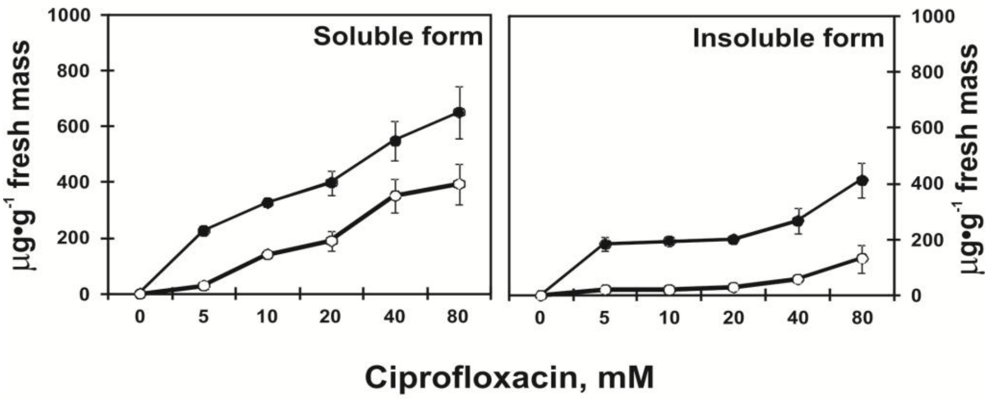

Figure 2. *Ciprofloxacin content in yellow lupin seedlings (○ - root, ● - shoot) growing eight days on soil supplemented with different concentrations (0, 5, 10, 20, 40, 80 mM) of CIP-HCl or free CIP base. Data points represent the means ± SD for three replicate samples.*

The roots and shoots of lupin growing in control soil contained 14.09 and 49.25 U of POD and 31.23 and 127.68 U of SOD, respectively (*Figs 3A, 3C*). The activity of peroxidase increased to 17.59 and 19.23 U in the roots of lupin growing in soil contaminated with the lowest of the concentrations of water-soluble and insoluble CIP (*Figs 3A, 3B*). The activity of the enzyme in roots was 78% lower than in the shoots of lupin exposed to water-soluble CIP (5-10 mM), and 74% lower after exposure to the insoluble form (*Figs 3A, 3B*). The activity of peroxidase in lupin shoots, regardless of the drug concentration, was similar. Its highest level was found in lupin shoots growing in soil contaminated with 5 mM CIP. An increase in POD activity of 38.22 and 31.55 U was recorded in samples growing in soil contaminated with the soluble and insoluble

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form of the drug, respectively. Both forms CIP at 20 mM concentration deactivated peroxidase in both the roots and shoots of lupin. The same tendency was observed for higher drug concentrations, i.e. 40 and 80 mM (*Figs 3A, 3B*). Unlike peroxidase, the activity of SOD in lupin shoots was inhibited by water-soluble and insoluble CIP even at the lowest, i.e. 5 mM concentration. The lowest concentrations of both CIP forms decreased the activity in shoots by 14.28 and 55.73 U, respectively. Shoots of plants growing in soil containing 80 mM of soluble CIP showed a higher SOD activity by 17 U compared with shoots growing in soil with insoluble CIP. The SOD activity in shoots of plants growing in soil containing the highest concentration of soluble or insoluble CIP (80 mM) was lower by 58% and 72%, compared with the control shoots (*Figs 3C, 3D*). On the other hand, in the roots, both forms of the drug at 10 mM concentration had a slightly stimulating effect on SOD activity. The highest SOD activity was detected in roots growing in soil containing 10 mM of soluble and insoluble CIP and it was 58.22 and 63.53 U, respectively.

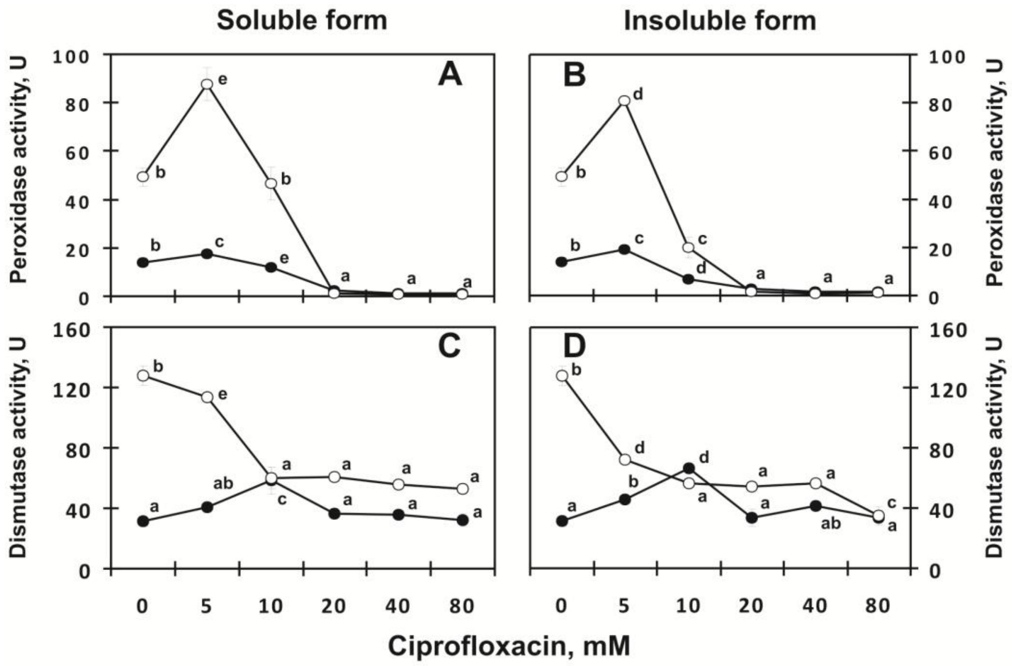

Figure 3. *Panel A and B peroxidase and panel C and D dismutase activity in yellow lupin seedlings (○ - root, ● - shoot) growing eight days on soil supplemented with different soluble and insoluble form of ciprofloxacin concentration (0, 5, 10, 20, 40, 80 mM). Data points represent the means ± SD for three replicate samples. Means with the same letter are not significantly different from each other (p≤0.01).*

Eight-day-old roots and shoots of lupin seedlings growing in soil contaminated with soluble or insoluble CIP were found to contain: D-*chiro*-inositol, D-pinitol, *myo*-inositol, galactose, glucose, and sucrose (*Figs 4A-F*). The content of D-*chiro*-inositol, D-pinitol, *myo*-inositol in control roots increased from 0.08, 0.07 and 0.34 mg×g^{-1} fresh mass to 0.54, 0.45, and 2.17 mg×g^{-1} fresh mass, respectively, in roots of plants growing in soil contaminated with 80 mM of soluble CIP (*Fig. 4A*).

The content of *myo*-inositol in roots growing in soil containing the highest concentration of insoluble CIP increased nearly four times. The roots contained an

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average of 10 times more glucose than galactose (*Fig. 4C*). The content of D-*chiro*-inositol and D-pinitol in lupin shoots was not modified by either form of CIP. On the other hand, content of *myo*-inositol increased two-fold with the highest of the CIP concentrations under study (*Figs 4A, 4B*). No galactose was found in the tissues of shoots growing in soil with insoluble CIP at concentrations from 5 to 20 mM, whereas the concentration of glucose increased, and that of sucrose remained unchanged (*Figs 4D, 4F*). Neither the soluble nor the insoluble form of the drug modified the content of D-*chiro*-inositol or D-pinitol. Both of the CIP forms at the highest concentration increased the content of *myo*-inositol by two-fold (*Figs 4A, 4B*).

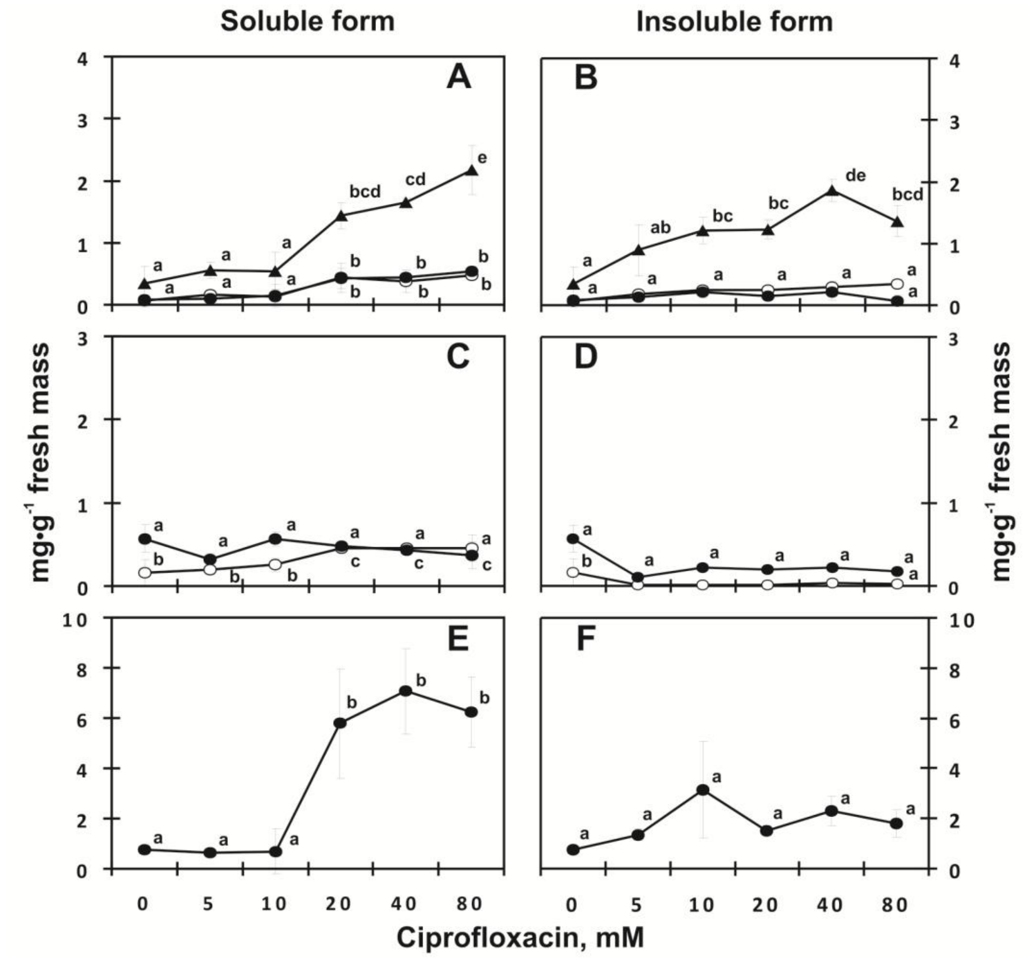

Figure 4. *Panel A and B D-chiro-inositol - ○, D-pinitol- •, myo-inositol-▲ content; panel C and D galactose -•, glucose - ○ content; and panel E and F sucrose-• content in roots of yellow lupin growing eight days on soil supplemented with different soluble and insoluble form of ciprofloxacin concentration (0, 5, 10, 20, 40, 80 mM). Data points represent the means ± SD for three replicate samples. Means with the same letter are not significantly different from each other (p≤0.01).*

Discussion

Fluoroquinolones are absorbed by organic matter in the soil and in manure, as a result of which they degrade slowly and thereby tend to persist in the environment (Kemper, 2008; Lillenberg et al., 2010). When present in the environment, they delay

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water supply to seeds and inhibit germination (Jin et al., 2009). In the present study, soluble and insoluble CIP at concentrations from 5 to 80 mM, did not inhibit lupin seed germination (*Figs 1A, 1B*). According to Jin et al. (2009), Adomas et al. (2013), Ziółkowska et al. (2014) and Piotrowicz-Cieślak et al. (2010) seed germination is not a good indicator of the presence of drugs in the environment. Elongation of roots can be a better measure of soil contamination with drugs. Low concentrations of drugs in soil have a positive effect on plants and can even stimulate plant growth (and hormesis occurs at low concentrations) (Migliore et al., 2003). Only 100-fold increase of enroflaxacin concentration (from 50 to 5000 $\mu g \times L^{-1}$) significantly decreases length of roots of plants such as cucumber (by 78%), lettuce (83%), beans (83%), and radish (66%) (Migliore et al., 2003). At the highest of the tested diclofenac concentrations (12 mM), the rate of root growth retardation compared with roots of control seedlings amounts to 85, 83, and 80% for pea, lupin, and lentil, respectively (Ziółkowska et al., 2014). Doxycycline at 25 µg/ml concentration inhibited root elongation of *Arabidopsis thaliana* seedlings by 67% (Moullan et al., 2015).

The presence of soluble or insoluble CIP in soil reduced the length of lupin roots and shoots (*Figs 1A, 1B*). Shoots are shortened on average by 91, 86, and 77% for pea, lupin, and lentil, respectively, exposed to 12 mM diclofenac (Ziółkowska et al., 2014). Shoots of lentil, soybean, adzuki bean, and alfalfa are shortened on average by 85% by 20 mM sulfamethazine (Piotrowicz-Cieślak et al., 2010). Drugs contaminating soil inhibit root elongation, on average by 78-84%, but the extent of inhibition depends on the kind of drug, its concentration, and exposure duration (Jin et al., 2009; Migliore et al., 2003; Moullan et al., 2015; Piotrowicz-Cieślak et al., 2010). Even though that the drug concentrations, compared with their lowest tested values, increased 100-fold (Migliore et al., 2003; Moullan et al., 2015), 200-fold (Ziółkowska et al., 2014), and 16 fold in our study, complete inhibition of root growth is not observed. It indicates that even high drug concentrations do not completely inhibit plant growth and development.

The shortening of the roots and shoots is accompanied by a reduction of their fresh mass (*Figs 1C, 1D*). Leaf biomass of barley and biomass of carrot roots are reduced as a result of exposure of plants to CIP in soil (Eggen et al., 2011). Similarly, oxytetracycline inhibits the biomass of alfalfa shoots and its roots (Kong et al., 2007). Plants growing in soil contaminated with drugs react to the inability of taking up water from soil with tissue dehydration, and consequently a decrease in the dry mass in the analysed under- and above-ground parts of plants. Dry mass of roots and shoots of lupin decreased with increasing concentrations of soluble and insoluble CIP (*Figs 1E, 1F*). The same was observed for dry mass of roots and shoots of narrow-leaved lupin growing in soil contaminated with enrofloxacin (Adomas et al., 2013), sulfamethazine (Piotrowicz-Cieślak et al., 2010) and common red soil contaminated with sulfamethazine, oxytetracycline hydrochloride and ciprofloxacin (Liu et al., 2013). It suggests that plants growing in soil contaminated with drugs take up less water. It may be a result of a different water potential between plants and the environment, which makes water uptake more difficult.

Drugs in the soil are absorbed by plants (Santos et al., 2010). The largest amounts of drugs accumulate in roots and leaves and the smallest amounts accumulate in shoots, as they only transport substances to leaves. Since the root is constantly exposed to contaminants, it takes up and accumulates the largest amounts of drugs (Liu et al., 2013). Eight-day-old lupin seedlings (their roots and shoots) took up soluble and insoluble CIP from soil, and CIP content in the plant tissues increased with increasing

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concentrations of the drug in soil. Roots accumulated more soluble and insoluble CIP than shoots. There was 1.5 times more soluble CIP in the analysed organs than its insoluble form (*Fig. 2*). Similar results were observed in an experiment conducted by Liu et al. (2013). Common reed takes up the largest amounts of soluble salts of drugs (sulfamethazine in the basic form and oxytetracycline and ciprofloxacin as hydrochloride), whereas the accumulation of drugs in plant tissues is positively correlated with their concentration in the substrate. The total content of sulfamethazine, oxytetracycline, and ciprofloxacin in tissues of wetland plant on day 62 of the experiment increased from 24, 165 and 345 ng×g^{-1} dry mass to 2047, 6901 and 13.843 ng×g^{-1} of dry mass, in the samples with the lowest and the highest level of contamination, respectively (Liu et al., 2013). It implies that content of drugs in plant tissues depends on the drug type, its concentration in soil and exposure time. Sulfamethazine content in plant tissues increases 85 times, oxytetracycline – 41 times, and ciprofloxacin 40 times. Mol mass of sulfamethazine, oxytetracycline and ciprofloxacin is 300, 460 and 330, respectively, and these values indicate that drug uptake does not depend solely on their mass (Eggen et al., 2011). It is assumed that drug accumulation is the most intensive when they are not too polar or highly hydrophobic. Drug dissolvability and bioaccumulation are defined by index Octanol-Water Partition Coefficient KOW. Log KOW values are generally inversely related to aqueous solubility and directly proportional to molecular weight (U.S. Environmental Protection Agency, 2009). In this study, we used two forms of CIP, soluble and insoluble, that differed in their speed of uptake from soil. We found more of the soluble form in lupin plants but the phytotoxic effect was the same as for the insoluble form. The mol mass for insoluble CIP is 385.82 and for soluble CIP it is 331.34. The log Kow for CIP is from 0.28 to 2.1 (Halling-Sørensen et al., 2000). Plants take up drugs from soil, using and by water transport and passive absorption Liu et al. (2013) are transported to roots, shoots, and seeds. High content of drugs oil seeds can be a risk for animals and people (Eggen et al., 2011).

Taking up and accumulation of drugs results in alteration of the course of numerous biochemical reactions in plants. It has been found that exposing plants to drugs results in inhibition of chlorophyll biosynthesis (Kong et al., 2007), decreases the concentration of carotenoids in leaves, inhibits photosystem II of photosynthesis (Zhu et al., 2001; Liu et al., 2013) and the production of abscisic acid (Pomati et al., 2004). These are not the only responses of plants to the presence of drugs in the environment. In legume plants treated with sulfamethazine, inhibition of root elongation was accompanied by changes in the activity of cytochrome oxidase in mitochondria and in cytosol. Lupin was particularly sensitive to the drug, which is indicative of its ability to detect soil contamination with sulfamethazine (Piotrowicz-Cieślak et al., 2010).

The activity of peroxidase in roots was lower by 78% on average than in lupin shoots exposed to soluble CIP (5mM), and by 74% when plants were exposed to its insoluble form (*Figs 3A, 3B*). Similarly, increasing concentrations of diclofenac in soil reduce the activity of cytochrome oxidase in root mitochondria of lupin, lentil and pea, with a simultaneous increase in the activity of the enzyme in cytosol (Ziółkowska et al., 2014). A change in the activity of antioxidative enzymes in leaves of sugar cane is also brought about by a mixture of sulfamethazine, oxytetracycline, and ciprofloxacin Liu et al., (2013). The highest concentrations of these drugs inhibit the activity of dismutase and catalase by 53% and 54%, respectively (*Figs 3C, 3D*). Although the activity of peroxidase increases at low concentrations, a larger amount of drugs reduces the activity of this

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enzyme. An increase of drug concentrations in soil and drug content in plants leads to a more effective antioxidative protection than in the control sample (Liu et al., 2013).

Deactivation of toxic substances and antioxidative protection (Watanabe et al., 2010) are not the only response of plants to soil contamination. The accumulation in plant tissues of compounds with many hydroxyl groups is also a common defence mechanism (Ortbauer and Popp, 2008). Plants react to changes of humidity and temperature by modification of the concentration of soluble carbohydrates, for example, raffinose family oligosaccharides (RFO) and polyhydroxyalcohols, such as their cyclic forms (cyclitols): *myo*-inositol, D-*chiro*-inositol, D-pinitol. Carbohydrates protect cellular structures and proteins with the hydroxyl groups in their structure, which can replace water (Adomas and Piotrowicz-Cieślak, 2004). RFO and galactosyl cyclitols are the basic forms of sugar storage in legume seeds, and their concentration does not usually exceed 20% of the plant dry weight. RFO is accumulated during natural or forced dehydration of seeds (Piotrowicz-Cieślak et al., 2003). Soluble carbohydrates are helpful in determination of the inhibition of germination and growth of plants (Ziółkowska et al., 2014), because complete decomposition of RFO and galactosyl cyclitols takes place during an early phase of lupin germination (Lahuta et al., 2000). The presence of drugs in soil also affects the content and metabolism soluble carbohydrates in plants. Seedlings of lupin, lentil, and pea exposed to diclofenac at 0.06-12 mM, accumulate D-pinitol, raffinose and stachyose, while containing less glucose, fructose and *myo*-inositol (Ziółkowska et al., 2014). Commonly occurring in plants, *myo*-inositol is an important substance in the process of plant growth. It is responsible for signal transduction in plants. Inositol is a precursor of phosphatidylinositol, phytic acid and it is a component of other compounds that take part in alleviating the effects of stress (Loewus and Murthy 2000). Pinitol in fully-hydrated legume plants is the main component of the fraction of soluble carbohydrates (McManus et al., 2000). However, even moderate water stress reduces its concentration, while fructose and sucrose increase their concentration. Sucrose is more effective for membrane protection than reducing sugars (Wang et al., 2013). The current study showed that 8-day old lupin seedlings take up and tolerate higher concentrations of soluble and insoluble CIP than concentrations found in the environment. Inhibition of root and shoot length is probably less harmful to the plants than the increased drug content in the plants that reduces enzyme activity (peroxidise and dismutase), and induces synthesis of D-*chiro*-inositol, D-pinitol and *myo*-inositol in seedling roots and shoots (*Fig. 4*).

Conclusions

The uptake of two forms of ciprofloxacin (water-soluble and insoluble) by plants was different. There was approximately 1.5 times more ciprofloxacin in seedlings treated with the water-soluble CIP compared to plants affected by the insoluble, free base CIP form. Both CIP forms were phytotoxic to lupin seedlings. Contrary to the preliminary assumptions, insoluble CIP inhibited elongation of roots and shoots nearly as severely as the water-soluble form. The study was carried out on reagent grade chemicals, so it depicts antibiotic-plant interaction in a simplified, reductionist model system. The patterns observed here might be confounded under real world, field conditions by partial transformation of the antibiotics in animal bodies and the presence of a plethora of accompanying molecules in manure.

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ROLE OF DIFFERENT PARAMETERS IN THE QUANTIFICATION OF GENERATED SLUDGE IN THE OXYLATOR UNIT OF WATER TREATMENT PLANTS, USING ARTIFICIAL NEURAL NETWORK MODEL (CASE STUDY OF JALALIEH WATER TREATMENT PLANT, TEHRAN, IRAN)

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Abstract. In recent years, smart imaging and error detection to achieve optimum performance in water and sewage treatment plants using Artificial Neural Network (ANN) detection technique has had a very active research field and has been regarded as one of the modern methods of modeling. This study has focused on predicting the sludge produced by the oxylator unit of the water treatment plant using an artificial neural network with multilayer Perceptron method. The measured input data: water flow, pH, turbidity, temperature, free residual chlorine, conductivity, consumed lime, coagulant (ferric chloride), and magnum and the sludge produced by the oxylator were examined as output data of the model in two consecutive years 2014 and 2015. At the end, the results of neural network analysis were used to show the effect of each of the parameters in the production of sludge. According to the results, turbidity and pH are the most effective parameters in predicting the sludge produced by the oxylator unit of the water treatment plant. This study indicated that artificial neural network could achieve a 0.99 validity with a regression of 0.9881 and mean square error of 0.006 among the observed and predicted output variables of the model. In addition, artificial neural networks provide an effective tool to analyze the data to understand and simulate the nonlinear behavior of treatment plants.
Keywords: *MLP, clarifier, disposal sludge, sludge quantity, solid wastage*

Introduction

A water treatment plant not only produces drinking water, but is also a generator (inverter and generator) of solid waste. Solid wastages of the treatment plant are generally produced in coagulation and flocculation ponds and backwash filter unit. Sludge management is one of the important issues in water and wastewater treatment plants, including two major objectives of minimizing the sludge production in the treatment plant and treating it, and its user programs. The sludge quantity and properties are two main parameters influencing the management of the methods and costs of sludge treatment and disposal (AWWA, 1981). Modeling many ecological processes that are the effects of the interaction of factors that are firstly high in number and secondly create a complex system through interacting with each other is a difficult but important issue. Simulation of processes of water and waste water treatment plants are not excluded from this rule and usually face problems. Most available models are almost based on assumptions

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and probability. Lee and Park (1999) simulated the three important stages of water treatment, that are units including coagulation, flocculation, sedimentation and coagulant injection tanks equipped with pump at a pilot scale, Satheesh et al. (2013) equipped it with a feed forward control and measured three qualitative parameters of water including turbidity, electrical conductivity and raw water pH as the input data and the alum required for treatment as the output data, and then compared the results of these two models by modeling the numbers in artificial neural network and fuzzy logic network separately in order to optimize the dosage of coagulant. Results for the values of R and MSE for validation data on the structure of NN, were 0.8861 and 0.3215 respectively in the structure of ANFIS, and 0.8310 and 0.1161. Sengul and Gormez (2013) using neural network TurboProp2 algorithms and data of Büyükçekmece treatment plant in Turkey, modeled the optimal dose of the coagulant. They used the NeuroShell, that normalizes the data and does not need the pre-processing of data. At first they modeled the water qualitative parameters in the initial model, and in the second model, they modeled the optimal dose of coagulant. R and MSE values were 0.95 and 0.02, respectively to predict pH, 0.7 and 0.038 to predict the turbidity, 0.93 and 13.05 to predict electrical conductivity, 0.47 and 1.83 for color parameter, 0.54 and 0.000006 to predict the UV parameter, and 0.19 and 156.67 to predict Aluminum. Noorani et al. (2013) modeled and examined the qualitative parameters of electrical conductivity and TDS of Zarrin Rood water treatment plant, using perspetron neural network. They selected parameters of temperature, turbidity, total hardness, pH and calcium as input to the network, and used FFNN algorithms for modeling. To announce optimum model results, they used the comparison with empirical methods to assess the electrical conductivity and TDS, and found out that empirical equations used to estimate the electrical conductivity is not able to estimate the different variations in temperature, while neural network resists well against these changes and provides optimal results compared to experimental equations. D and RMSE results arising out of them for TDS output, with a structure of 1-5-5, are respectively, 0.81 and 0.028, and for outgoing EC, with a structure of 1-5-5, 0.73 and 0.037, respectively, and for TDS and EC output with a structure of 2-7-5, 0.74 and 0.059, respectively and for TDS output, with a structure of 1-3-1, is 0.98 and 0,007, respectively. Rak (2012) using Perspetron artificial neural network predicted the turbidity of Sosnówka water treatment plant in Poland with input data to models that include turbidity of raw water, tank input water, water level, daily rainfall and tank input water temperature and achieved the desired riteria of R=0.84 and RMSE = 0.49.

CP (clean production) Methods, are integrated approaches to handling wastages and pollution in industries. (Visvanathan and Kumar, 1999). In addition, the importance of CP, has motivated leaders of different countries at different levels of industrial development to adopt national strategies and plan to accelerate the implementation of CP rules (Ghzinoory and Huisingh, 2006). CP principle represents the continuous proactive environmental approach to reduce pollution at the source of production. In addition, the ability of clean production methods is in developing clean systems and creating social and economic benefits (Zarkovic et al., 2011; Giannetti et al., 2008). Management of solid waste from a public health perspective as well as the social, economic and industrial function is very important, because with the constant increase in the quantity of production of dangerous or non-dangerous materials, they should be disposed safely and economically and preferably, with a recycle approach. Large amounts of waste water treatment materials result from drinking water treatment plants every year (Huang et al., 2005). Cleaner production has allowed industrial production to find a place in this vision by recasting negative impacts of polluting industrial processes and products into positive images of news technologies that are materials-conserving, energy- efficient, nonpolluting and low-waste, and that produce ecologically friendly products, like which are responsibly managed throughout their lifecycle (Geiser, 2001).

Materials and methods

The supply source of Jalalieh treatment plant is Karaj River in Beilaghan Basin. Raw water after removing sand and large brooms and preliminary chlorination in the basin is transferred to the treatment plant by two lines of steel pipes with a diameter of 1000 mm and a length of 40 km in gravity manner. Physical and microbiological treatment is performed along with some chemical filtration (refinement and reduction of some gases and heavy metals). Oxylator is a clarification unit under acceleration, which works based on mud workflow. In this clarifier, sludge is kept by the mixer in suspension and in circulation.

Artificial neural networks are information processing models that are inspired from the human brain neural networks. These networks consist of a large number of interconnected processing elements that work with each other in harmony. The idea of artificial neural networks began with the study of human brain cells. The idea of artificial neural networks began with the study of human brain cells. Human brain is made up of millions of unique neurons and these nerve fibers change in different shapes and sizes. Artificial neural networks consist of different parts. Components of an Artificial Neural Network include:

Input signals X1 to Xn, are the equivalents of neural input signals and make up the total neuron input. The input, shown with vector X, can be the output of other layers.

Weight vector Wi1 to Win weights are the equivalents of neuron input synaptic connections values. The effect of input Xi on output y is measured by weight feature. Wi is adjustable and is moderated based on transformation functions and type of learning algorithm.

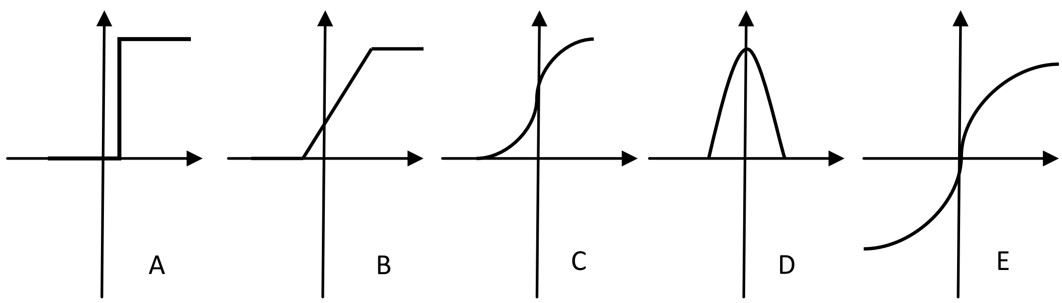

Figure 1. *A) Threshold Function, B) Multi Piece Linear, C) Sigmoid, D) Gaussian, E) Hyperbolic Tangent*

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Aggregation Function defined by the following equation, performs the neuron processing. In one-neuron networks, the aggregation function to some extent determines the output and in multi-neuron networks, the aggregation function determines the level of activity of the neuron j in the inner layers.

$$\text{Net j} = \sum_{j=1}^{n} Wij\ Xi + bi \qquad \text{(Eq.1)}$$

bi: refers to bias, that is actually like a weight, except that its input is a constant 1 (Sun et al., 2016).

Activity Function also called compression function or transformation function; a function that determines the artificial neuron output values. Activation function maps a wide range of input values to a specific amount of output. There are various types of compression functions (*Fig. 1*).

Compression, hyperbolic tangent and sigmoid, functions are used the most. These functions are calculated through the following relationship (Funes et al., 2015):

$$\text{Sigmoid function:}\quad \mathrm{P(t)} = \frac{1}{1+e^{-1}} \qquad \text{(Eq.2)}$$

$$\text{Hyperbolictangentfunction:}\quad Y = \frac{e^{x} - e^{-x}}{e^{x} + e^{-x}} \qquad \text{(Eq.3)}$$

Output refers to the response to the problem. The neuron output is determined by the following function:

$$Yi = \text{ActivationFunction}\left(\sum_{j=1}^{n} Xj\ \ Wij\right) \qquad \text{(Eq.4)}$$

Artificial neural network training process depends on several components and hence, a concept should be defined as network performance measurement. Then, the rules according to which the network performance should be set must also be defined. *(Fig. 2)* displays a schematic of the education network.

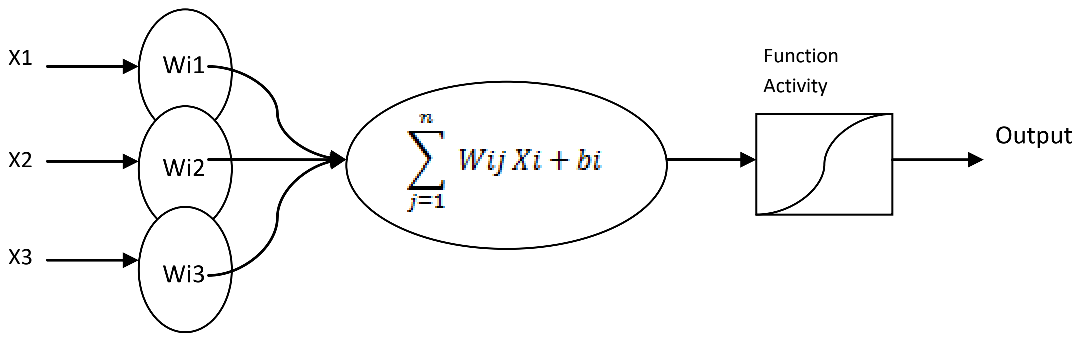

Figure 2. *The structure of an artificial neurons*

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Neural networks can be classified based on the concept of measuring network performance. When the network performance function is based on error measurements, the training is deemed as supervised. Normally, error is defined as the difference between network output and the favorable predefined response (Muthupriya et al., 2011). Common indices used to measure the performance of neural networks are given below (Djeddou and Achour, 2015). In all equations, T represents the actual output, $\overline{T}$ represents the average actual output of the network, O represents the network output, $\overline{O}$ represents the average network output and N is the total number of samples used.

Mean Square Error $$MSE = \frac{1}{N}\sum_{i=1}^{N}(Ti - Oi)^2 \quad \text{(Eq.5)}$$

Root Mean Square Error $$RMSE = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(Ti - Oi)^2} \quad \text{(Eq.6)}$$

Mean Absolute Percentage Error $$MAPE = \frac{1}{N}\sum_{i=1}^{N}\frac{|Ti - Oi|}{Ti} \times 100 \quad \text{(Eq.7)}$$

Absolute Relative Error $$ARE = \frac{1}{N}\sum_{i=1}^{N}\frac{|Ti - Oi|}{Ti} \quad \text{(Eq.8)}$$

The sum of squared errors $$SSE = \sum_{i=1}^{N}(Ti - Oi)^2 \quad \text{(Eq.9)}$$

Regression coefficient $$R = \frac{\sum(Ti - \overline{T}i)(Oi - \overline{O}i)}{\sqrt{\sum_{i=1}^{N}(Ti - \overline{T}i)2 \sum_{i=1}^{N}(Oi - \overline{O}i)2}} \quad \text{(Eq.10)}$$

Coefficient of determination $$R^2 = 1 - \left[\frac{\sum_{i=1}^{N}(Ti - Oi)^2}{\sum_{i=1}^{N}(Ti)^2}\right] \quad \text{(Eq.11)}$$

Clasifications done on different structures of artificial neural networks are based on feedback and advance criteria. If at least one ring can be found in the network, in which one neuron returns to itself or to the past layer, the network is characterized as feedback. Feedback networks have more potential than advance networks and can better show the system time-related behavior characteristics. If in a neural network, the output of each neuron is connected only to the neurons of the next layer, it is deemed as advance. Advance neural network is also known as fully connected neural network, if each cell in each layer is connected to all the neurons in the input layer. But if some synaptic connections are removed, the resulting network is called partly connected *(Fig. 3)*.

Multilayer Perceptron is one of the most commonly used types of neural networks (Vyas al., 2011).

In general, regarding the classification of staticpatterns, a Multilayer Perceptron with two medial layer, is used the most. In other words, separation functions can be used in these networks in any form.

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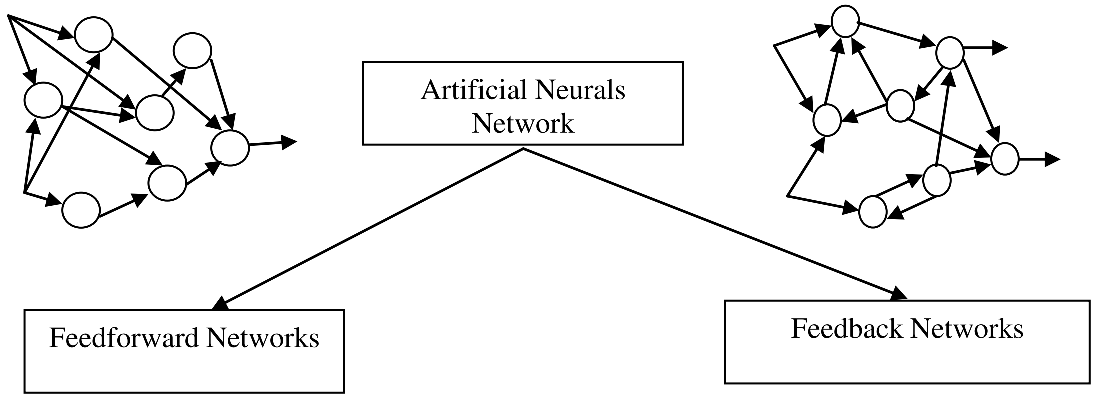

Figure 3. *Netwok of feedforward or feedback*

Results and discussion

The recorded data of water flow rate, input turbidity, temperature, electrical conductivity, pH, lime consumption, free residual chlorine, the coagulant consumption and the contribution of the consumed coagulant was received during 22 consecutive months from operation control unit of the company. In addition, the daily production of sludge was extracted using the software SCADA by the operation unit. The Kolmogorov - Smirnov test that determines the normal distribution for the continuous quantitative variables was used with spss software version 19. The results indicate normality of sludge variables, free residual chlorine and the remaining pH, while other variables are not normally distributed. For the normal results of the data, Pearson test was used for the correlation of the normal data, and Spearman correlation coefficient was used for non-normal data. In order to analyze the input data, the correlation between different parameters with each other as well as various parameters with the sludge produced by the oxylator unit was conducted using SPSS version 19. Pierson and Spearman's correlation coefficient was used because of the relativity of the variables used in this study *(Table 1)* (Cronbach, 1951).

Table 1. *Correlation coefficient matrix of variables*

	Flow	Turbidity	Temprature	Ec	pH	CaCo3	Cl_2	$FeCl_3$	Magnom	sludge
Flow										
Turbidity	-0.214	1								
Temprature	0.463	-0.132	1							
Ec	-0.17	-0.046	-0.062	1						
pH	0.093	0.135	0.099	-0.113	1					
$CaCo_3$	0.213	-0.022	-0.189	0.331	0.007	1				
Cl2	-0.468	-0.234	-0.274	0.185	-0.066	0.011	1			
$FeCl_3$	0.325	-0.059	-0.24	0.25	-0.037	0.893	-0.032	1		
Magnom	0.488	-0.258	0.115	-0.131	-0.278	0.008	-0.278	0.139	1	
sludge	-0.01	-0.003	-0.253	0.09	-0.111	0.163	0.001	0.187	0.063	1

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Different networks in terms of the number of hidden layer neurons and activation functions were created. Results of the table show that the correlation coefficient of lime consumption variable with ferric chloride is very high. Hence, by entering the variable values of the lime used as input data, the neural network model does not receive new data. Therefore, this parameter is ignored in the present simulation. Results of descriptive figure of the data used in the model are shown in *Table 2*.

Table 2. *Matrix of statistical descriptions of model variables*

	Min	Max	Mean	Std.Dev.
Flow	107800	24000	211547.46	16745.13
Turbidity	1.1	8.4	2.88	1.12
Temprature	6.3	33.6	13.34	3.81
Ec	231	1020	505.19	73.58
pH	7.45	8.5	8.09	0.15
$CaCo_3$	277	928	685.24	104.45
Cl_2	0.34	1.09	0.69	0.14
$FeCl_3$	774	2318	1740.09	222.59
Magnom	0	3	2.17	1.16
sludge	74	216	139.21	35.53

In this study, the most commonly used method, which is the Levenberg-Marquardt training method, was used. In order to display the Network training, structure of *(Fig. 4)* was used.

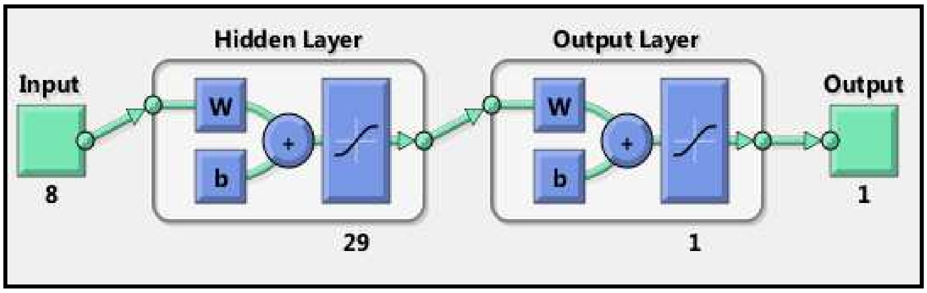

Figure 4. *Structure of network training*

Fig. 5 shows the results of network training to analyze regression coefficients in different structures are 1 to 30 neurons. Activation functions tansig- tansig are given in the order from left to right in the middle layer and output layer. The results including the correlation coefficient (R), the mean squared error (MSE) and root mean square error (RMSE) for each of the three categories (training,validation and testing) for each of the neurons in the middle layer are separated.

Fig. 6 shows network learning results to analyze error coefficients in different structures are 1 to 30 neurons.

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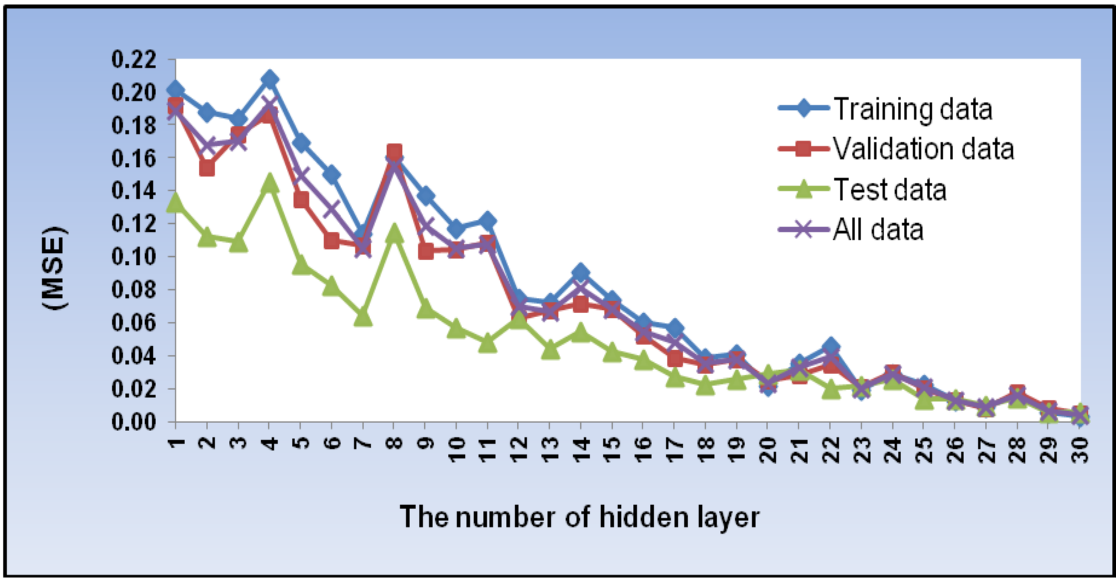

Figure 5. *Analytical diagram of regression coefficients in training validation and testing data, for activities functions tansig-tansig*

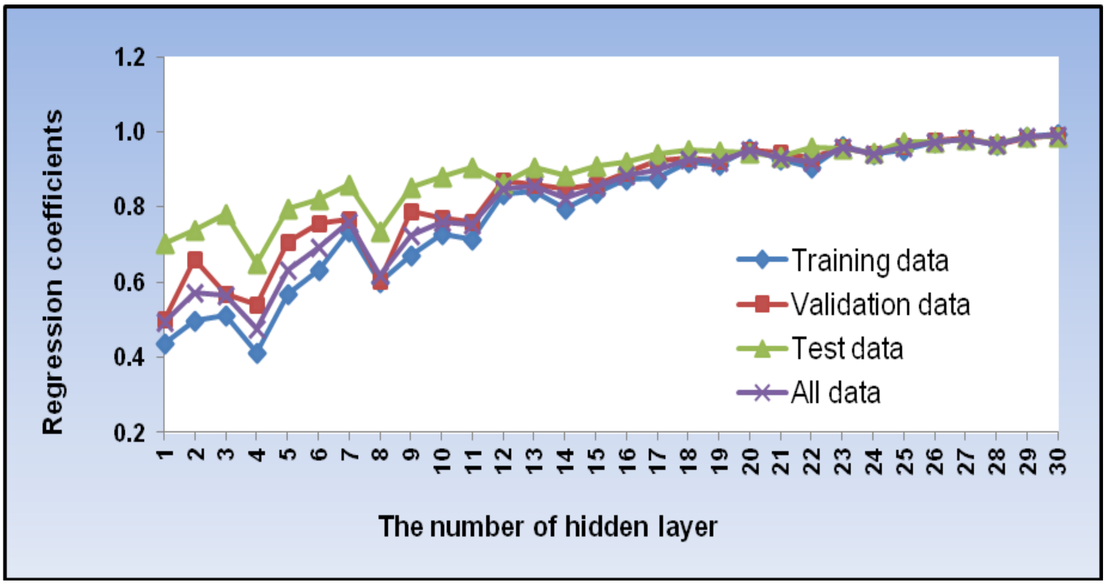

Figure 6. *Analytical diagram of error coefficients in training validation and testing data, for activities functions tansig-tansig*

Finally, the optimal number of neurons to predict network with the lowest error 29 neurons with activation functions tansig-tansig, is the desired choice for the network. Diagrams and values of regression and network error are according to *(Fig. 7), (Fig. 8), (Fig. 9)* and *(Fig. 10)* for all data, education data, validation data and test data related to the optimum network. For each of the main data and pre-processed data during network:

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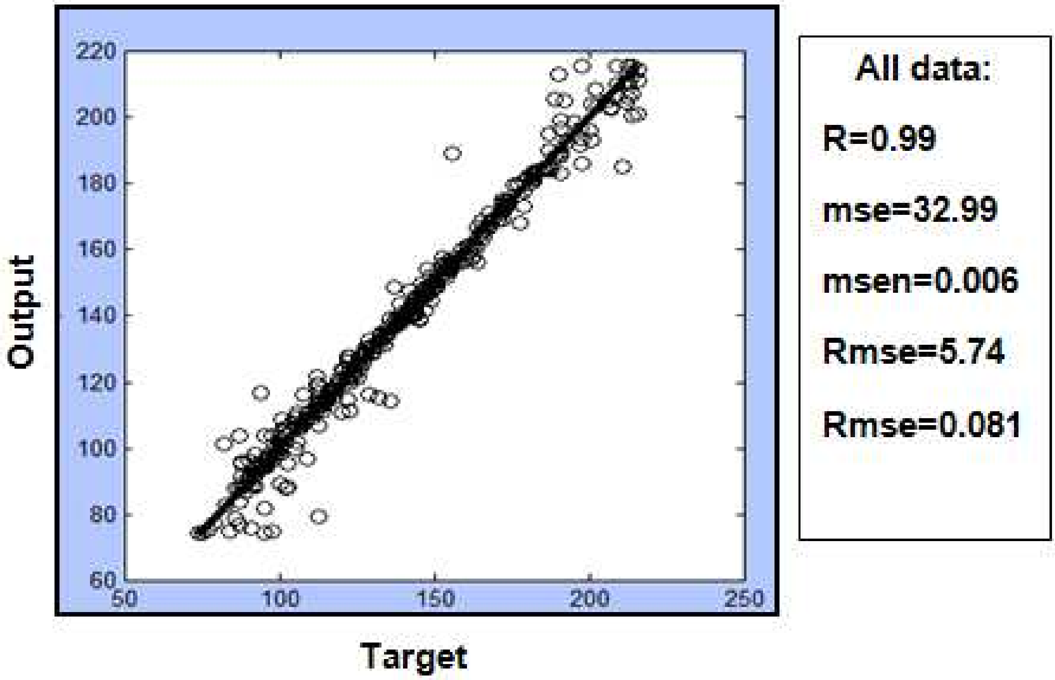

Figure 7. *Values of R - MSE - RMSE for all data set*

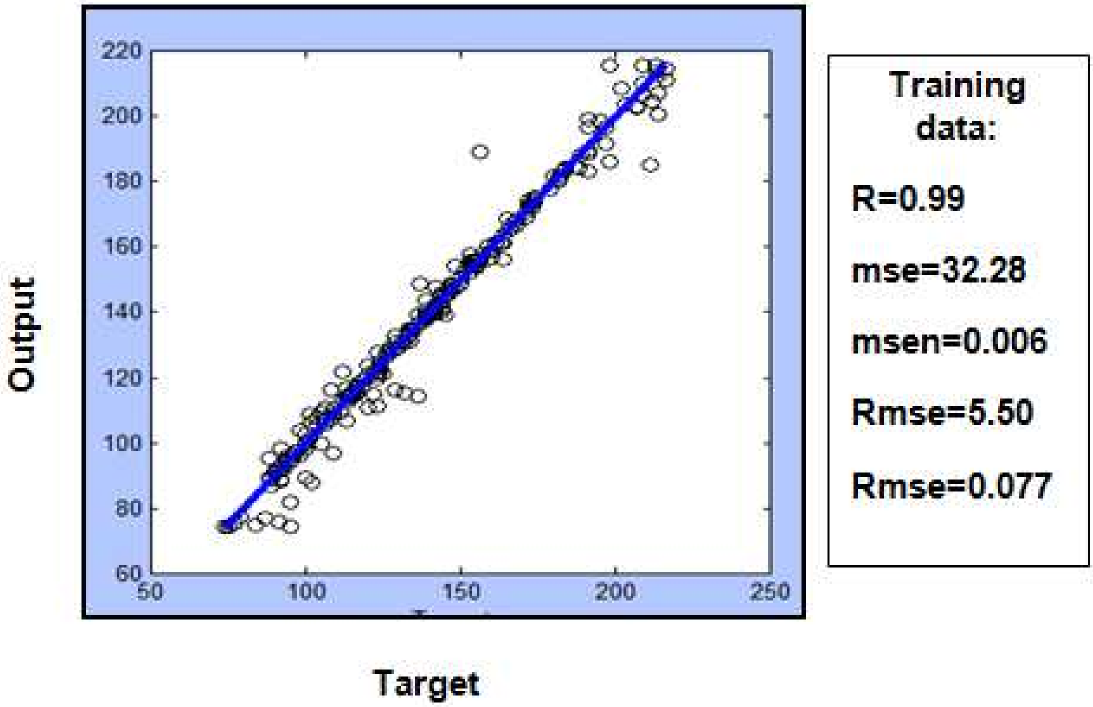

Figure 8. *Values of R - MSE – RMSE for training data set*

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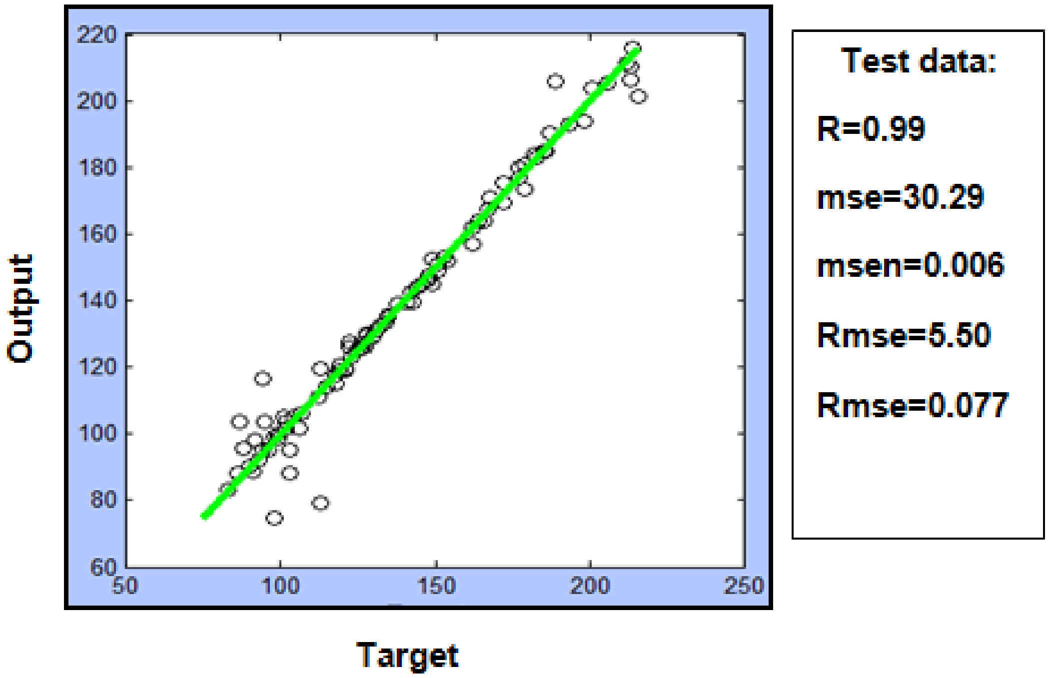

Figure 9. *Values of R - MSE - RMSE for test data set*

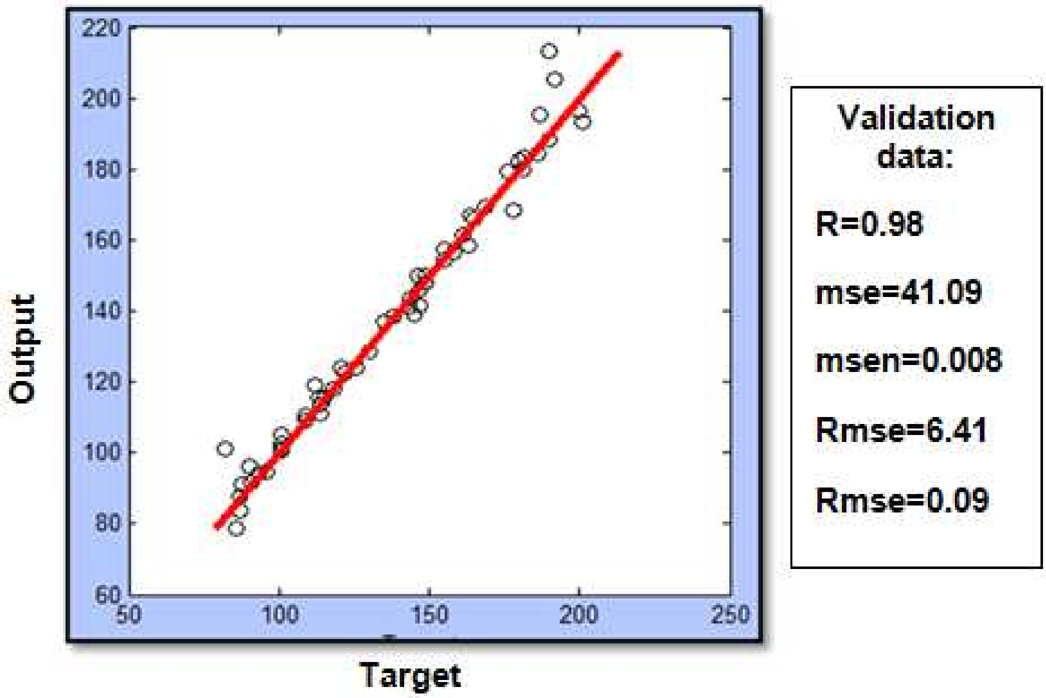

Figure 10. *Values of R - MSE - RMSE for validation data set*

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Since the artificial neural network considers a number of data to learn the networks and some data and some data in order to avoid duplicate network training mode,that is the network validation phase, it also chooses some data in order to extend the network, which is network test data. Model prediction is shown in *(Fig. 11)*.

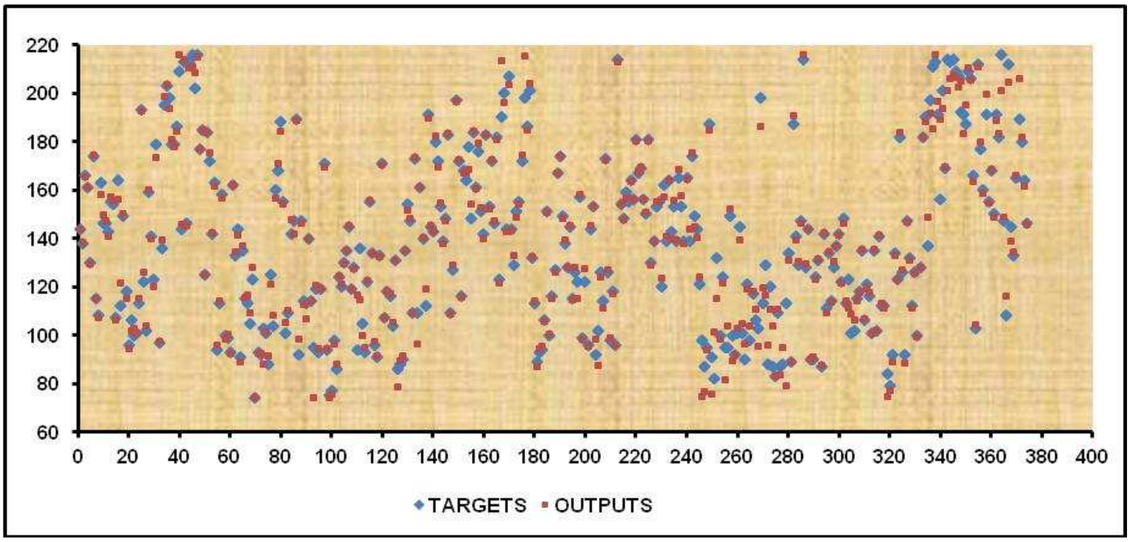

Figure 11. *The results of model prediction against the actual values of the treatment plant*

Sensitivity analysis allows the level of accuracy required for a parameter to be considered so that the model is valuable and useful enough. Sensitivity analysis can also be used to determine what parameter values are reasonable to be in the model (Vilimek, 2014). In this study, because of the complex environmental, physical and chemical process of factors in the treatment plants and the non-linear behavior among them, a decrease or increase in one does not necessarily lead to increases or decreases in output variable. Hence, in order to investigate the role of each of the input parameters in the estimation of the produced sludge, the impact of weight between neurons in the input intermediate and output layer was used in the calculations, according to formula (12) (Montana and Palmer, 2003):

$$Ij = \frac{\sum_{j=1}^{Nh}\left[\frac{|Wij|}{\sum_{r=1}^{Ni}|Wrj|}|Wjk|\right]}{\sum_{i=1}^{Ni}\left[\sum_{j=1}^{Nh}\left[\frac{|Wij|}{\sum_{r=1}^{Ni}|Wrj|}|Wjk|\right]\right]} \quad \text{(Eq.12)}$$

where, Ni is the number of neurons in the input layer,
Nh is the number of neurons in the middle layer,
Wij is the weight between neurons in the input layer and the middle layer (weight between input neurons i and the hidden neuron j),
Wjk is the weight between neurons in the middle layer and output layer (weight between hidden neuron j and the output neuron k),

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$\sum_{r=1}^{Ni}|Wrj|$: The sum of all weights between the ith input neurons and jth hidden neuron.

The results are provided in *(Table 3)*.

Table 3. *Results of sensitivity analysis on the input data*

Input data	I_j
Turbidity	31.41
pH	30.83
Temprature	15.19
EC	10.91
$FeCl_3$	4.09
Magnom	2.99
Flow	2.32
Cl_2	2.25

As can be seen from the table results, the most effective parameters according to the weighting coefficients between layers, in estimating the daily sludge production of water treatment plant, are, input water turbidity, pH, temperature, electrical conductivity and the coagulant used in the process, respectively.

Conclusion

Given the diverse world-wide applications of sludge from water treatment plants in recent years, which can be used in many different industries, with the approach of clean production methods in order to develop clean systems and to create economic and social benefits for societies, including the production of light-weight bricks by combining the sludge resulting from water treatment plant and rice bran in creating green and eco-friendly future buildings, (Chianga et al., 2009), use of sludge produced by water treatment plants in the structure of clay products such as clay bricks can also be a good alternative instead of clay used in their composition (Hegazy et al., 2012). Use of the sludge from the water treatment plant as an alternative to the cement used in the manufacture of tiles and paving slabs can lead to a significant reduction in the production costs of the industry and also provides a healthy and environmentally friendly option for disposal of sludge from water treatment plants (Wolff et al., 2014). According to various studies on the use of sludge from water treatment plants, it is necessary to accurately predict the daily quantity of the sludge from a water treatment plant with the process governing it. So, it is recommended to use artificial neural network as a powerful mathematical model capable of modeling and creating nonlinear relationships between independent and dependent variables. The results (high regression and low grid error) as well as the self-evaluation and self-monitoringcapability of this software toward generalizability and to prevent its overfitting shows the high capability of artificial neural network as a powerful instrument to predict daily values of sludge produced by water treatment plant oxylator unit. This means, having high functionality and technology has a considerable speed in data analysis. Neural Network Models are

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used for prediction and classification in cases where classic statistical methods cannot be used because of their limitations. Artificial Neural Networks can be used for modeling water and sewage treatment plants process because of their high accuracy and perfect application in engineering (Neelakantan, 2001).

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CO_2 EMISSIONS FROM PAKISTAN AND INDIA AND THEIR RELATIONSHIP WITH ECONOMIC VARIABLES

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Abstract. The rapid increase in CO_2 emissions has been a hot topic for whole world because of their major contribution to greenhouse gases (GHG) which is an ultimate cause of global warming. This study analyses spatially gridded data from EDGAR (Emissions Database for Global Atmospheric Research) and linear relationship using multiple linear regression model between CO_2 emissions and four economic variables (energy use, urban population, gross capital formation and GDP at market prices) for Pakistan and India. Additionally, four major tools (f-test, t-test, time series analysis, and prediction errors) are used for the purpose of investigating linear relationship and efficiency of the model. EDGAR data shows that about 200 teragram CO_2 has been emitted from Indo-Gangatic plain. Analysis revealed that the most effective predictor for both the Pakistan and India is energy use. The value of f-stat and t-stat showed that the economic variables have joint and individual significance for the regression model at $p<0.05$. Time series revealed that CO_2 emissions increased gradually from 1971 to 2011. Error analysis indicated that regression model for Pakistan is more efficient than that of India. New policies can be devised and decisions can be taken on the basis of information given by this paper.
Keywords: *GHG, Energy use, EDGAR, prediction errors*

Introduction

Economic activities have been increased in the world since the last century. These activities not only promoted the countries in economic development but made an immense increase in CO_2 emissions also. Human activities are adding over 29 billion tons of CO_2 to the environment each year (Goodal, 2007). Contribution of CO_2 to the total greenhouse gases (GHG) is reported to be 58.8% (World Bank, 2007). This rapid increase in CO_2 is so dangerous that it can lead to the severe situations like storms, flood, and other environmental calamities. CO_2 emissions have also caused the sea level to rise to 10-20 cm (Mukhtar, 2004).

It has been noted that due to 30% overall increase in CO_2, the global temperature has also risen by 32.54-33.08 °F (Spence, 2005). There are also some other elements which are contributing to the drastic environmental changes i.e. population, transportation, economic growth, energy consumption and industrial activities (Abdullah, 2015).

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Numerous researches have been conducted to study the relationship among these variables, which clearly indicate the importance of the topic for the whole world (Moran and Gonzalez, 2006; Lozano and Gutierrez, 2007; Ang, 2009; Su et al., 2009; Freitas and Kaneko, 2011; Keat et al., 2015). Bi-variate and multi-variate methods have been used by numbers of researchers to study the relationship between CO_2 and economic variables (Abdullah, 2015).

Four variables; CO_2, population, gross domestic product (GDP), and energy consumption, have been used by Lozano and Gutierrez (2007) through a non-parametric frontier approach to investigate the association between these variables for USA. The study finds a positive relationship between the estimated variables. Moran and Gonzalez (2006) used the methods of linear programming to find the major productive linkage between CO_2 emissions and human activities. This study also found a direct relationship between the variables. Freitas and Kaneko (2011) studied the links between CO_2 emissions and economic growth in Brazil. They concluded that energy intensity, economic activity and demographic pressure increase the emissions. Data for more than 50 years was used by Ang (2009) in order to explore the determinants of CO_2 emissions for China. This study reported a negative relationship between CO_2, research intensity, technical transfer and absorptive capacity in china. On the other hand, a positive relationship was found between energy use, high income, trade openness and CO_2 emissions. Numbers of studies have been conducted to test the comparative relationship of CO_2 emissions and economic variables. Su et al. (2009) used the data of China and Singapore to study the relationship of CO_2 emission and trade. The study concluded that in 40 sectors export is positively correlated with carbon emissions. Martinez and Maruotti (2011) applied STIRPAT model for 88 developing countries from 1975 to 2003 in order to investigate the relationship of CO_2 and urbanization, which showed an inverted U shaped relationship of concerned variables, which means positive relationship existed between urbanization and carbon emissions at 10% level. Aqeel and Butt (2001) determined that the energy consumption is the determinant of economic growth for Pakistan. Energy consumption and pollution has positive relationship for both long and short term (Shahbaz et al., 2010). In an analysis, Keat et al. (2015) examined the correlation between petroleum products and CO_2 emissions by using multiple linear regression model. They found high correlation value (R^2 = 0.9544) among the variables. Mohiuddin et al. (2016) found positive relationship of CO_2 emissions with three parameters i.e. energy consumption (EC), GDP, and electricity production from oil, coal and natural gas over Pakistan.

The trend of carbon dioxide emissions is rising alarmingly in South Asian countries (i.e. India, Pakistan, Bangladesh, Sri Lanka and Nepal) (Sarker et al., 2013). Due to rapid increase in urbanization and industrialization, Pakistan and India are experiencing high amounts of greenhouse gases and air pollutants. Several studies, employing different techniques such as satellite and ground based remote sensing, and *in situ* measurements, have been conducted to monitor greenhouse gases and air pollutants over the sub-continent (Ali et al., 2014; Tariq and Ali, 2015; Tariq et al., 2015; Tariq et al., 2016; Ul-haq, 2014; Ul-Haq, 2015a; 2015b; 2015c; 2015d; Badarinath, 2009). Ang (2009) focused on China, a growing economy, and found that an increase in energy use and trade intensity contributes to higher CO_2 emissions in china. However, in this paper an effort has been made to find the relationship of CO_2 emissions with four economic variables (energy use, urban population, gross capital formation and GDP at market price) over two south Asian countries. Only those economic variables have been

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considered that are significant ($p<0.05$) determinant of CO_2 emissions in both Pakistan and India. To the best of our knowledge no study has so far been conducted to estimate linear relationship between CO_2 emissions and economic indicators of energy use, urban population, gross capital formation, and GDP at market price for Pakistan and India. The focus of this study is to investigate the relationship between CO_2 emission and the four economic variables for Pakistan and India.

Materials and Methods

Datasets

To investigate spatial distribution of CO_2 emissions we used total CO_2 emissions from EDGAR (Emissions Database for Global Atmospheric Research) version 4.2 data over Pakistan and India during the period 1970-2008. EDGER is a globally gridded database of anthropogenic emissions of greenhouse gases and air pollutants from all the sectors. Same type of economic variables was taken for both the countries for the purpose of easy comparison. Data of CO_2 emissions and four economic variables (energy use, urban population, gross capital formation and GDP at market prices) is sourced from the world data indicator (World Bank, 2016). Data of all the variables were obtained from 1971 to 2011 for both the countries.

Linear relationship of predictors and response variables

Multiple linear regression (MLR) model is used to study linear relationships between the variables. To get interdependencies for a given dataset, the model is widely used to describe the link between the response and predictor variables (Keat et al., 2015). Multiple regressions analysis is most common tool being used in the field of sciences for understanding the results (Courville et al., 2001; Nimon et al., 2010; Zientek et al., 2008).

MLR model is called linear because it forms a straight line when plotted over graph. The functional form of Simple Linear Regression (SLR) is usually written as follows:

$$Y = m + bX \qquad \text{(Eq.1)}$$

where Y is a dependent or response variable for a selected value of X. m represents the Y-intercept and b is a slope of the line. The above equation shows the relationship between one response and one predictor variable. However, there may be a situation in which we need to investigate the relationship of one response variable with multiple predictors. For such kind of relationship MLR model can be used. MLR model is used when we have to study the relationship between one response and several predictors. In MLR model the additional predictor variables (denoted by X1, X2, X3, …., and so on) are used to study the relationship between a dependent and multi independent variables. The general form of MLR equation is:

$$Y = \mathrm{m} + \mathrm{n1X1} + \mathrm{n2X2} + \mathrm{n3X3} + \cdots..\mathrm{nkXk} \qquad \text{(Eq.2)}$$

where m is the Y-intercept and k is any positive integer which is used to represent numbers of predictors in MLR equation. "n_k" is the slope of Y i.e a unit change in n_k with the corresponding change in Y.

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Analysis and Results

In order to understand the spatial distribution of CO_2 emissions, we have plotted total CO_2 emissions (see *Figure 1*) from EDGAR data over Pakistan and India during the period 1970-2008. It can be observed from the *Figure 1* that high amounts (about 200 terra gram) of CO_2 have been emitted from Indo-gangatic plain (in north and north eastern parts of India) during the period 1970 to 2008. These high emissions are due to rapidly growing urbanization, industrialization, economic activities and biomass burning. It can further be noted that north eastern parts of Pakistan and almost entire India have emitted almost 20 terra gram of CO_2 in the period from 1970 to 2008.

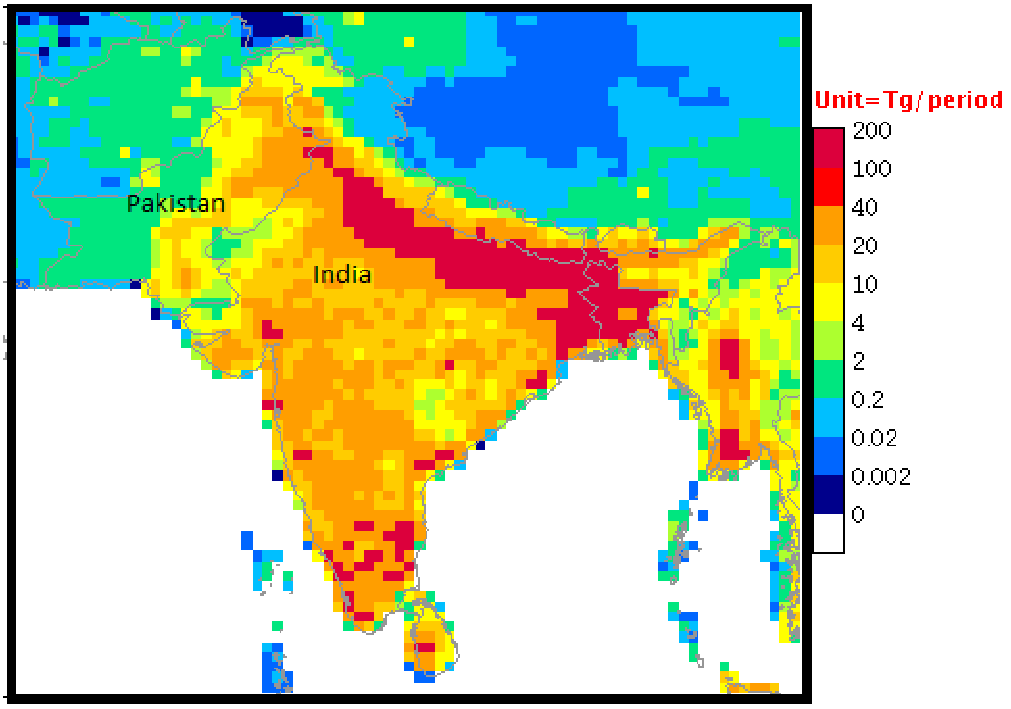

Figure 1. *Spatial distribution of total CO_2 emissions over Pakistan and India in the period 1970-2008.*

Data of CO_2 emissions and economic variables of two developing countries i.e Pakistan and India have been taken for the purpose of studying the linear relationship among them. Data for CO_2 emissions and economic variables have been considered for the period 1971 to 2011 for both the countries. Same type of economic variables was taken for both the countries for the purpose of easy comparison. Data for both dependent and independent variables have been taken from the official website of World Bank (World Bank, 2016). Predictors for Pakistan as well as for India data are energy use, urban population, gross capital formation, GDP at market prices, which are represented as E1, U1, C1, and G1 respectively for Pakistan, whereas the variables for India data are denoted by E2, U2, C2, and G2 respectively. CO_2 emission is a dependent variable which is denoted by Y1 for Pakistan and by Y2 for India. For study purpose the analysis section is divided into four parts which are explained below.

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Excluded predictors

The four predictors that were considered for regression analysis for both India and Pakistan have been shown in the *Tables 1 and 2*. Tables show all the predictors with their corresponding t-values and p-values for Pakistan and India. Regression coefficient analysis shows that all predictors for Pakistan are significant and therefore included in full model because they are significant at 95% level because probability of acceptance is defined at $p<0.05$.

Table 1. *Predictors for Pakistan data*

Model	t-value	Sig(p-value)
Energy Use (E1)	5.043451516	1.31549E-05
Urban Population (U1)	2.706522425	0.010328845
Gross capital formation (C1)	-2.570265226	0.014445252
GDP at market price (G1)	6.4717461	1.63514E-07

Table 2. *Predictors for India data:*

Model	t-value	Sig(p-value)
Energy Use (E2)	6.122963685	4.76218E-07
Urban Population (U2)	3.311246823	0.002120419
Gross capital formation (C2)	-0.977359963	0.334913416
GDP at market price (G2)	1.287407878	0.206167702

Table 2 shows that predictors E2 and U2 are significant only because their significance value (p-value) is less than 0.05 whereas predictors C2 and G2 are insignificant because their significance value (p-value) is more than 0.05. Thus, the predictors C2 and G2 should be excluded from the model of India.

Analysis of variance

After exclusion of insignificant predictors, the selected variables were used to model CO_2 using MLR technique. *Tables 3 and Table 4* show summary results for the MLR model for Pakistan and India data respectively.

Table 3. *MLR test for Pakistan data*

Regression Statistics	
Multiple R	0.997723762
R Square	0.995452705
Adjusted R Square	0.99494745
Standard Error	3376.590289
Observations	41

From the regression statistics of Pakistan, value of R^2 and adjusted R^2 is found to be 0.995452705 and 0.99494745 respectively pointing towards the fact that about 99%

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variation in CO_2 emission is explained by energy use, urban population, gross capital formation and GDP at market price for Pakistan. This analysis of variance shows that the P-value for the F-test statistics is 3.47144E-47, which is providing strong evidence to reject the null hypothesis. The *Table 4* shows the MLR model for India data.

Table 4. *MLR test for India data*

Regression Statistics	
Multiple R	0.998204755
R Square	0.996412733
Adjusted R Square	0.99622393
Standard Error	33354.1464
Observations	41

ANOVA (Analysis of Variance)

	Df*	SS**	MS***	F****	Significance F
Regression	2	1.17E+13	5.87E+12	5277.51	3.47E-47
Residual	38	42274965109	1112499082		
Total	40	1.17E+13			

* *stands for Degrees of freedom, ** Sum of squares, *** Mean square, **** F statistics*

From the regression statistics of India data, the value of R^2 and adjusted R^2 is found to be 0.996412733 and 0.99622393 respectively indicating that about 99% variation in CO_2 emission is explained by energy use, and urban population for India. ANOVA indicates that the p-value for F-test statistics is 3.47144E-47, which is strong evidence against the null hypothesis. It is also indicated by ANOVA that degree of freedom is 2, indicating minimum number of independent variables for complete system. Sum of square is found to be 1.17×10^{13} which is showing deviation from mean. The ratio of sum of square to the relevant degree of freedom called mean square and is found to be 5.87×10^{12}. It is clear from the above discussion that there exists a linear relation between predictors and CO_2 emission.

Multiple regression equations

Regression coefficients for all the predictors for Pakistan are given in *Table 5*. From the above table it is clear that p-value and coefficients are against rejection region having p-value less than 0.05. It is providing a strong evidence for rejecting then null hypothesis. These results indicate that these four economic variables are good predictors for CO_2 emissions for Pakistan.

Table 5. *Regression coefficients for Pakistan*

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-63984.7977	8847.997555	-7.23155689	1.63498E-08	-81929.3683	-46040.227
Energy use	262.5086441	52.04940372	5.04345152	1.31549E-05	156.947561	368.069727
Urban Population	0.001005508	0.000371513	2.70652243	0.01032885	0.00025205	0.00175897

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Gross capital formation	-877.477516	341.3957077	-2.57026523	0.01444525	-1569.8601	-185.09493
GDP at market prices	2.65923E-07	4.10898E-08	6.4717461	1.63514E-07	1.82589E-07	3.4926E-07

The regression equation given below shows that E1 is the best predictor for CO_2 emission in Pakistan. MLR equation for above regression coefficient for Pakistan data is given in equation 3.

$$Y1=63984.79+262.50\ E1+ 0.0010\ U1-877.47\ C1 +0.00000026\ G \qquad \text{(Eq.3)}$$

Table 6. *Regression coefficients for India*

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%
Intercept	-1346910.76	44694.4021	-30.136006	3.8908E-28	-1437389.9	-1256431.7
Energy use	5110.86089	323.755895	15.7861555	2.8098E-18	4455.45136	5766.2704
Urban population	0.00130128	0.00033639	3.86840378	0.00041641	0.00062030	0.0019822

It can be seen from the *Table 6* that p-value for energy use and urban population for India data is less than 0.05, which is a clear indication that these two variables are significant for India. Thus, we can reject the null hypothesis. Therefore we can conclude that the better predictors for CO_2 emissions for India are energy use and urban population. MLR equation of India for above regression coefficients are given in equation 4.

$$Y2 = 1346910.76 + 5110.86\ E2 + 0.0013\ U2 \qquad \text{(Eq.4)}$$

The above equation is showing that E2 is the best predictor for CO_2 emission in India. The value of f-stat and t-stat showed that the economic variables have joint and individual significance for the regression model at $p<0.05$. It can be noted from the regression equations for Pakistan and India that the best predictor against CO_2 emissions is energy use. Our findings are partially consistent with the findings of Wang et al. (2011, 2016) and Ang (2008).

For comparison, the time series data of actual and modeled CO_2 emissions have been plotted together in *Figure 2*. It can be noted from the *Figure 2(a)* that CO_2 increased gradually from 1971 to 2011 over Pakistan. It can further be observed from the figure that actual and modeled CO_2 over Pakistan show a similar pattern indicating the efficiency of regression model. The values of actual CO_2 emissions ranged from 18929 to 163452 while modeled values of CO_2 emissions ranged from 15103.50 to 173441.50. The mean value of actual CO_2 emissions was found to be 77446.32 while mean value of modeled CO_2 emissions is found to be 77446.33 which are very close to each other. It is noted from the *Figure 2(b)* that CO_2 emissions are increasing steadily from 1971 to 2011 for India. Similar pattern of actual and modeled CO_2 is observed which indicates the competence of regression model. The mean value of actual CO_2 emission for India was found to be 849817.23 while mean value of modeled CO_2 emissions is found to be 849817.21. The lowest and highest modeled CO_2 values are 172859 and 2096057

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respectively. The minimum and maximum values of actual CO_2 emissions are 205869 and 74344 respectively. It can also be observed from *Figures 2a* and *2b* that actual and modeled CO_2 emissions of India (2074344) are greater than the corresponding CO_2 emissions of Pakistan

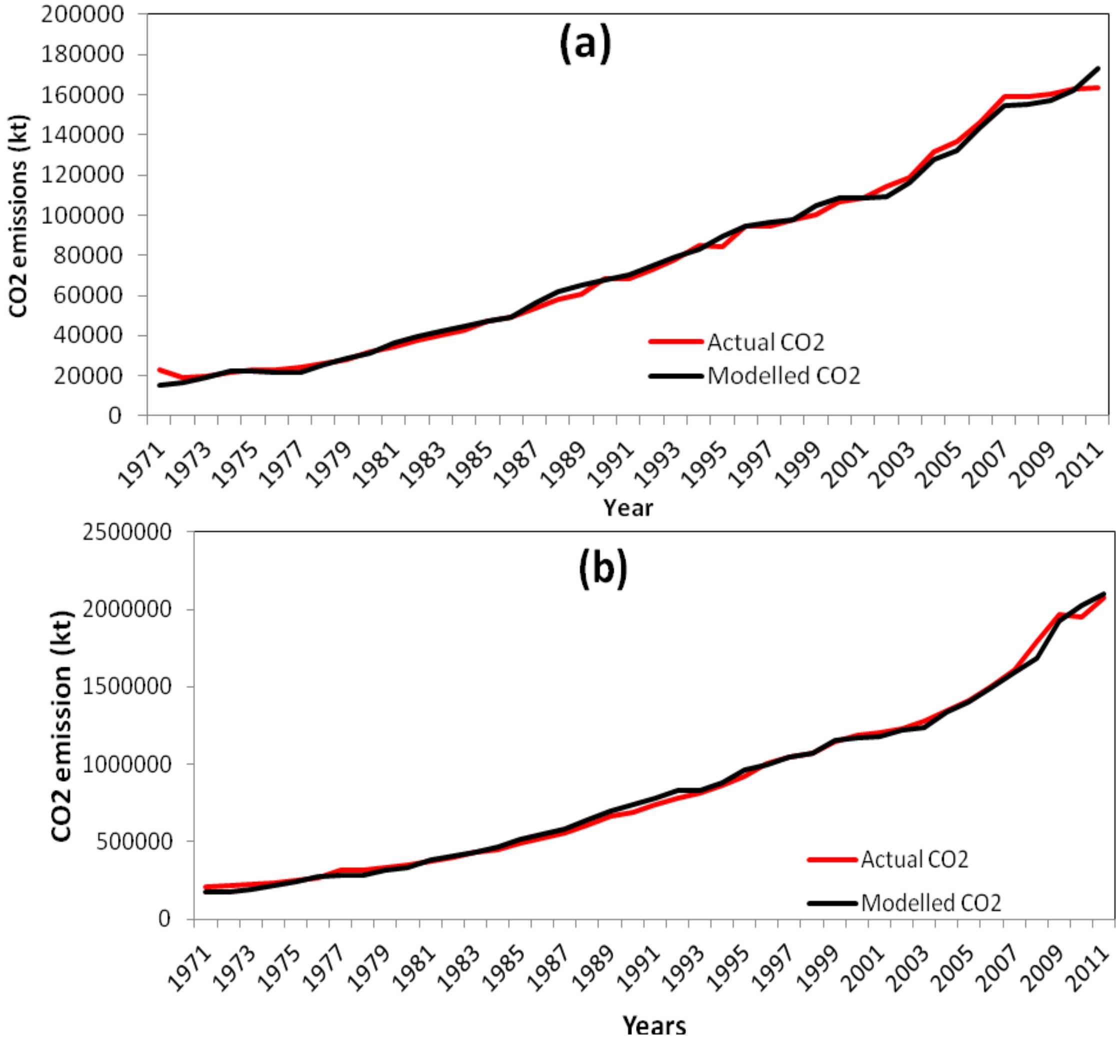

Figure 2. *Time series of modeled and actual CO_2 emissions over (a) Pakistan and (b) India*

Prediction errors

Performance of predictors in the regression model can be measured using two techniques known as MAE (Mean Absolute Error) and RMSE (Rout mean square error). These measures are used to show that how predicted values are performing against the actual values. The difference of actual value and predicted values is called prediction error or deviation error. Equations used to measure predicted error are given below:

$$MAE = \frac{1}{n}\sum_{t=1}^{n} \left|\frac{At - Pt}{At}\right| \quad \text{(Eq.5)}$$

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$$RMSE = \sqrt{\frac{1}{n} \sum_{t=1}^{n} (At - Pt)^2} \quad \text{(Eq.6)}$$

In equations 5 and 6 *At* is representing the actual value, *Pt* is representing predicted value and *n* is representing to the maximum numbers of values in a selected model. *Table 7* is showing the error results of both Pakistan and India model.

Table 7. *Errors for Pakistan and India model*

Model	MAE	RMSE
Pakistan data	0.00676	3164.0088
India data	4.99452	2694012.884

It is quite clear from the above table the model of Pakistan has less error than the model of India. This means that regression model of Pakistan is more efficient than that of India. Pakistan is a populous country and the use of petroleum and natural gas is increasing with the increase in population. A considerable amount of carbon is being emitted from natural gas consumption for electricity use (Alam et al., 2007). Less efficiency of regression model of India is might be due to the fact that predictors considered in this study for India are not enough for prediction of CO_2 emissions. Use of other variables may produce better results for India and is recommended for future research. Furthermore, the prediction of CO_2 emissions in this study may not be consistent with the research conducted at sectoral level data. The use of sectoral data is outside the scope of this study and left for upcoming research.

Per-capita energy consumption (kW h) values of India and Pakistan are 644 and 457 respectively. Higher value of per-capita energy consumption (kW h) for India indicates fast economic growth of India as per-capita energy consumption is proportional to economic growth of a country. India has electricity generation of 1193.48 TW h with 17% share from renewable energy sources while Pakistan has electricity generation of 97.796 TW h having 36% share from renewable energy sources (Ahmad et al., 2016). Contribution of coal in electricity production is much higher in India as compared to Pakistan. India is 3rd largest emitter of CO_2. For both countries solar energy is a good choice as it is cleanest energy source and can help in sustainable economic growth. The overall approximate solar energy potential of India and Pakistan are 5000 trillion kW h and 2900 GW h respectively (Ahmad et al., 2016). Due to poor political infrastructure and the lack of research and development centers, Pakistan had been unable to promote sustainable development goals (Ang, 2008). However, presently Pakistan is paying serious attention towards the use of solar energy and in this regard several projects are underway such as Quaid-e-Azam solar energy park of 1000 MW capacity in Bahawalpur, 300 MW solar plant in Quetta and 3000 solar home systems in Tharparkar (Ahmad et al., 2016). Moreover, twenty two projects with the capacity of 772.99 MW are being installed in the country. Similarly, India has also started solar energy projects such as National Solar Mission (NSM) for installation of 22 GW. During 2015–16, 827.22 MW of solar energy was added to the system that enhanced the installed capacity up to 4579.24 MW (Ministry of New and Renewable Energy India, 2015). Other clean renewable energy sources such as wind, hydro and geothermal are also being installed in both the countries.

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Conclusions

In this paper, the multiple linear regression model has been used to investigate the relationship between CO_2 and economic variables for Pakistan and India. The time series of CO_2 emissions for Pakistan and India showed an increasing trend over time especially from 2004 to 2011. Out of the four selected predictors (energy use, urban population, gross capital formation and GDP at market price) only two predictors, energy use and urban population were found to be significant ($p<0.05$) for India, however all the four variables were found to be significant ($p<0.05$) for Pakistan for the prediction of CO_2 emissions. CO_2 emission model for Pakistan showed that four economic variables (E1, U1, C1 and G1) are contributing 99% of the CO_2 emissions in Pakistan. On the other hand, CO_2 emission model for India showed that out of four variables (E2, U2, C2, and G2) only two variables (E2 and G2) are contributing 99% of the CO_2 emissions in India. The regression equation indicated that energy use is the most effective predictor for both Pakistan and India for CO_2 emission. Results from the error of prediction indicated that regression model for Pakistan is more efficient than that of India. Thus, predictors on CO_2 emissions for India are not enough to be considered in prediction of CO_2 emissions. The prediction of CO_2 emissions in this study may not be consistent with the research conducted at sectoral level data. The use of sectoral data is outside the scope of this study and left for upcoming research. Spatially gridded data from EDGER shows that about 200 terra gram CO_2 has been emitted from Indo-Gangatic plain (in north and north eastern parts of India) over the period 1970 to 2008. The results suggest the use of renewable energy resources to reduce the CO_2 emissions. There is a need to enhance the use of clean energy for both countries for sustainable development. On the basis of the information given in this paper new policies can be devised and decisions can be taken.

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FRAGMENTED LANDSCAPES OF EAST BOKARO COALFIELDS: A REMOTE SENSING BASED APPROACH HIGHLIGHTING FORESTLAND DYNAMICS

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Abstract. Due to massive mining practices and subsequent spurt of human habitation, the East Bokaro coalfield region of Jharkhand in India has faced enormous changes in the landscape. This article aims to study the land cover changes and fragmentation dynamics of the landscape especially forests from 1972 to 2016. Remote sensing based satellite images combined with spatial analysis were used to derive the land cover and fragmentation dynamics over the last 44 years. The results reveal the existence of six primary land cover types namely agriculture, forest, water bodies, mining, settlement and barren land/scrubland. Agriculture and forest which are the dominant categories decreased while mining, settlements and barren lands increased at the expense of forests and agriculture. Fragmentation analysis brings out significant trends in landscape changes that have occurred from 1972 to 2016. The most prominent fragmentation metrics were observed for forests and agriculture classes. Agricultural land has continuously been converted and fragmented into other classes. The forests also show strong fragmentation during 1972-2001 period but in 2016 the level of fragmentation is not very high. This study is a preliminary step towards evaluating the long term impact of mining and its related activities on the landscapestructure.
Keywords: *land cover, coal mining, fragmentation, landscape metrics, forests*

Introduction

A landscape refers to all the visible features of a region of land and their integration with the man made features. Landscape is mostly studied on the basis of composition and configuration of the spatial arrangements of different ecosystems that it is composed of (Tolessa et al., 2016). Composition refers to all the land cover attributes or habitats while configuration refers to the various levels of fragmentation of those habitats. Changes in the composition and configuration of landscapes form the basis of landscape dynamics and fragmentation studies. Fragmentation studies integrated with land cover analysis paves the way for detecting and understanding the spatial patterns of landscape changes (Jansen et al., 2002). There is a lot of research to support that most of the changes in landscapes are brought about by the anthropogenic activities and fragmented landscapes are an indicator of this conspicuous interplay between mankind and the environment (Dupin et al., 2013).

Forests are a very important constituent of the landscape due to their critical role in maintaining biodiversity, regulating climate and providing essential ecological resources. Hence the loss of forest cover from the land raises global concerns on

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biodiversity, climate change and ecosystem services. However, losing forest cover is not the only concern. Traditional methods document the area of forest lost but the crucial part that needs to be studied is the spatial distribution and fragmentation of the forests and the way these fragmented forests affect the functioning of the ecosystem. Studies on the forest and human health impacts of coal mining are very poorly represented in literature (Upgupta and Singh, 2017). There are a lot of international level studies which have done landscape level fragmentation work for example, Southworth et al. (2002), Armenteras et al. (2003), Millington (2003), Echeverria (2006), Dupin et al. (2013), Tolessa et al. (2016). According to Ritters et al. (2002), fragmentation not only deals with the quantity of habitat loss but also the spatial connection between patches of the habitat. Landscape fragmentation research has mostly been conducted on forests (Cakir et al., 2007) and the majority of forest fragmentation studies have a prime focus on the wildlife habitat as compared to the trees. Various scientists in India have studied forest fragmentation and its impacts on the species diversity like Roy and Tomar (2000) in northeastern India; Jha et al. (2005) in the Vindhyas; Malaviya et al. (2010) in Central India; Behera (2010) in the eastern Himalayas. Roy and Joshi (2001) used remote sensing to study the fragmented landscapes of northeast Indian Himalayan Region. The study emphasized the importance of shape, richness and diversity indices in evaluating spatial distribution of land cover and concluded that fragmentation has led to the lapsed connectivity, corridors and vanishing ecotones as well as meta-populations. De and Tiwari (2008) did research on the Rajaji-Corbett National Parks using LISS III datasets and remote sensing software. They estimated the patchiness of different forest types in the national Parks. The forests of Himalayan foothills were studied by Munsi et al. (2010) using satellite remote sensing and fragmentation analysis. They quantified the landscape structure using landscape based metrics. Giriraj et al. in 2010 used fragmentation to study the forests communities of Southern Western Ghats. The developmental activities like building roads for better connectivity and increasing resource utilization for economic upliftment of local people leads to fragmentation. Therefore fragmentation studies are very important when evaluating changes in forested habitats over a period of time.

Numerous tools are available to estimate a number of fragmentation metrics from remote sensing classified data on land and forest cover. Fragmentation metrics can be computed using different software platforms e.g., FRAGSTAT, SPLAM and Landscape Fragmentation Tool. One of the most widely used and applied tool is the FRAGSTAT which was developed by McGarigal and Marks (1995) to quantify fragmentation. FRAGSTAT gives elaborate statistical information of a variety of landscape levels. Spatial Landscape Modeling (SPLAM) gives landscape parameters like patchiness, interspersion, porosity, juxtaposition, fragmentation and landscape modeling. Landscape Fragmentation Tool maps the types of fragmentation present in a specified land cover type (i.e. forest). It also creates value-added map layers that can be used to quantify and assess the amount and type of fragmentation present in a landscape and runs on ArcToolbox through a Python script. Metrics can be computed at the class level, patch level as well as the landscape level for the whole study area. However class-level metrics are most frequently used in ecological studies as they measure the distribution of one particular habitat within a landscape.

The East Bokaro coalfield region is a predominantly mining area with coal as the major mineral resource being mined. The area was rich in biodiversity and had a large

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forest cover with fertile cultivable patches. With the advent of coal mining and industrialization, the region witnessed rapid increase in population, economic growth and urbanization. Due to this the original land cover and its rich vegetation cover underwent rapid degradation and the region is subjected to high levels of disturbance and environmental degradation. The goal of this research is to study the changes in land cover during 1972 to 2016 and to assess as how coal mining triggered development and urbanization are driving the fragmentation of the landscape especially forests.

Materials and Methods

Study area

The study area is located in the Bokaro district of the Indian sub-continent. It lies between 23^0 45’ to 23^0 50’ N latitude and 85^0 30’ to 86^0 03’ E longitude (*Figure 1*). It covers an area of 259 sq. km with elevation ranging from 230-300m above mean sea level (MSL). The general slope of the area is from west to east and the area is marked by Lugu Hill (1070m) which is a prominent landmark and also the separation point between the East and West Bokaro coalfields. Tenughat reservoir in the southern part is a tourist attraction. Bokaro thermal power plant is the major industrial set up. This coalfield is in the form of a long narrow strip extending for about 64 km in an east-west direction with a maximum width of about 11 km in the north-south direction.

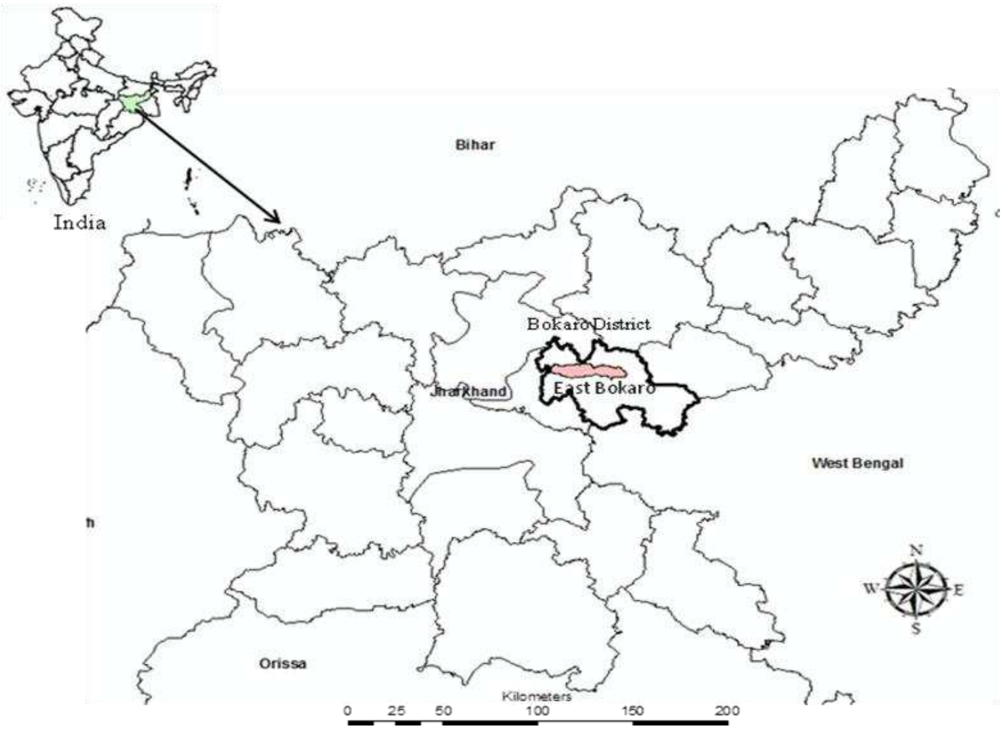

Figure 1. *Location map of East Bokaro, Jharkhand*

The Barkar Formation is the chief coal bearing horizon. Damodar and Bokaro are the primary rivers draining the landscape from north to south. Geologically, the northern part of the area is composed of massive sandstone formation which has resulted in a

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rugged topography. The East Bokaro is a source of medium coking coal in India. Coal is produced in East Bokaro by underground as well as opencast mining methods by Central Coal Field limited (CCL) a subsidiary of Coal India Limited. The important coal seams found in the coalfield include Jarangdih seam, Kargali seam, Bermo seam and Karo seam.

Climatically, the area has an average temperature of 30 °C during summer season while 20 °C during the winter season. During summer, the maximum temperature rises up to 44 °C while during winter the minimum temperature comes down to 2°C. On an average, the area receives about 1200 mm of rainfall with the bulk of rainfall occurring around the July-September period.

Currently 28.1% of the total geographical area of East Bokaro is under forest cover. Majority of these forests belong to the Tropical Dry Deciduous forest type group. There is 16.6 sq.km, 36.9 sq. km and 21.6 sq.km of the forests having very dense (>70% canopy density), moderately dense (40–70% canopy density) and open forests (10–40% canopy density), respectively [FSI, 2013]. The Bermo subdivision of the area is comparatively rich in forest wealth and the forests are replete with Sal (*Shorea robusta*) along with other trees like mango (*Mangifera indica*, Shisham (*Dalbergia sissoo*, jackfruit (*Artocarpus heterophyllus*) and kendu (*Diospyros melanoxylon*).

Data used

Multi-temporal satellite images of different time periods representing long term and discernible changes were acquired for this analysis. For land cover mapping moderate spatial resolution Landsat images of three time periods 1972 (Landsat 1 reprocessed, Multi-Spectral Scanner), 2001 (Landsat 7, Enhanced Thematic Mapper plus) and 2016 (Landsat 8, Operational Land Imager) at 57m, 28.5m and 30m were used. These images were downloaded from the Earth Explorer USGS image database (Landsat Imagery Archive). The images were chosen based on their availability for the study area, the defined time periods, clarity of data and the season of data capture. Cloud free images were collected for the same season for the month of November 2016. Landsat images are already geo-referenced and were subjected to appropriate atmospheric corrections in the next level of image preprocessing steps to bring out more clarity in the interpretation of the land cover features.

Methods

All the analyses were performed in ArcMap environment. The data for the three time periods were extracted for the study area using the Clip tool in ArcMap 10.2. The delimiting boundary of the East Bokaro coalfield was acquired from the Central Coalfields Limited. FRAGSTAT 4.2 was employed to carry out fragmentation analysis and estimate the various class level metrics.

Land cover classification

Supervised Maximum Likelihood Classifier (MLC) approach was used for land cover classification followed by cleaning of the classified images using majority filters. Six classes namely agriculture, forest, water bodies, mining, settlement and barren/scrub were identified. These classes were based on the land cover classification system developed by National Remote Sensing Centre, Hyderabad in the Manual of Nationwide Land Use/ Land Cover Mapping Using Satellite Imagery" (NRSC, 2006). Majority

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filters were used to get rid of very small group of pixels below a threshold value of 10 pixels. Ground verification was conducted in the same season as that of satellite image collection. Random points were generated and for every point the existing class in the ground and the corresponding land cover type in the classified image were noted. Accuracy assessment of the classified map of 2016 was performed in Arc Map 10.2 using pivot tables.

Fragmentation analysis

Classified land cover raster datasets were first converted to tiff file format and then used as the inputs into the fragmentation analysis. FRAGSTAT4.2 was used to carry out fragmentation analysis and estimate the various landscape and class level metrics. Landscape metrics were computed at the class level for all the mapped land cover classes of agriculture, forest, water bodies, mining, settlement and barren/scrub. Based on the recently reviewed literature and forest fragmentation studies, the following twelve landscape metrics (*Table 1*) were selected for quantification and comparison.

Table 1. *Landscape metrics used in the analysis*

Level of Analysis	Metric Name	Abbreviation	Description	Valid Range	Units
Class Level	Percentage of landscape	PLAND	Percentage of total landscape area occupied by the largest-sized patch	0 < PLAND < 100	%
	Patch Density	PD	Number of patches on a per unit area		-
	Number of Patches	NP	Total number of patches in the class	N ≥ 1	-
	Largest patch Index	LPI	Percentage of total landscape area occupied by the largest-sized patch of each class	0 < LPI < 100	%
	Mean Patch Size	AREA_AM	Average of patch size in hectares		m2
	Edge Density	ED	Sum of length of all edge segments for the class, divided by total landscape area	ED ≥ 0	m/ha
	Euclidean nearest neighbor	ENN_AM	Mean of minimum edge-to-edge distances to the nearest neighboring patch of the same type of a certain class	ENN ≥ 0	m
	Shape Index	SHAPE	Measures the complexity of patch shape of a particular class	SHAPE≥1	-
	Perimeter to area ratio	PARA	Measure of patch shape	SHAPE≥1	-
	Fractal Dimension	FRAC_AM	Index of the complexity of shapes on the landscape	1 ≤ FRAC ≤ 2	-
	Aggregation Index	AI	Percentage of neighboring pixel of the same class, based on single-count method	0 ≤ AI ≤ 100	%
	Interspersion Juxtaposition Index	IJI	Measure of evenness of patch adjacencies		

These metrics have been widely applied and are the best for the evaluation of spatial properties of fragmented landscapes. Although the analytical outputs were reported for all the land cover classes, forest category was specially emphasized on the spatio-temporal pattern of forest fragmentation during 1972-2016.

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Results

Land cover dynamics

The land cover maps representing the spatial pattern of the six identified cover classes of East Bokaro coalfields for the different time periods that is 1972, 2001 and 2016 are shown in *Figure 2*. The area statistics for all the identified classes and for three time periods are shown in *Table 2*. The accuracy assessment for the classified map of 2016 using the ground verification points yielded an overall accuracy of 81% and kappa co-efficient of 0.7. The error matrix for accuracy assessment is shown in *Table 3*.

Table 2. *Comparison of areas and change statistics of Land cover classes between 1972 and 2016*

Sl. No.	Land Cover	1972		2001		2016		Change (1972 to 2016)		Rate of change
		Area (sq.km)	Area (%)	Area (sq.km)	Area (%)	Area (sq.km)	Area (%)	Area (sq.km	Area (%)	(ha/yr)
1	Agriculture	121.48	46.89	114.02	44.08	87.04	33.59	-34.44	-28.35	-78
2	Forest	95.48	36.86	85.46	33.04	92.49	35.70	-2.99	-3.13	-7
3	Water Body	14.02	5.41	13.28	5.13	13.72	5.29	-0.3	-2.14	-1
4	Mining	9.64	3.72	13.36	5.16	19.23	7.42	9.59	99.48	22
5	Settlement	7.74	2.99	20.39	7.88	27.71	10.70	19.97	258.01	45
6	Barren/Scrub	10.69	4.13	12.18	4.71	18.90	7.30	8.21	76.80	19
	Total	259	100	259	100	259	100			

It can be observed from the statistics provided in *Table 2* that in the year 1972, 36.86% of the land was covered by forests and this decreased to 33.04% in the year 2001. But the next decade of 2016 is marked by an increase in forest cover to 35.70%.

Since 1972 to 2016 there is an overall decline of 3.13% in the total forested areas. Similar trend is observed in the water bodies but the change observed is not very significant (*Table 2*). Water bodies also show a decrease in coverage followed by a slight increase. The overall decrease stands at 2.14% since 1972 to 2016.

Table 3. *Error matrix of* overall *accuracy*

Classification	Ground Truth Classes								
	Agriculture	**Forest**	**Water Bodies**	**Mining**	**Settlement**	**Barren/Rocky**	**Sum Total**	**Commission**	**User Accuracy**
Agriculture	26	1	0	0	2	8	37	33.33%	66.67%
Forest	0	37	0	1	0	0	38	2.6%	97.40%
Water Bodies	0	0	4	0	0	3	7	42.9%	57.10%
Mining	0	1	0	3	0	1	5	40%	60%
Settlement	0	0	0	0	5	0	5	0%	100%
Barren/Rocky	0	0	0	0	0	8	8	0%	100%
Sum Total	26	39	4	4	7	20	100		
Omission	0%	8%	0%	25%	43%	60%			
Producer Accuracy	100%	92%	100%	75%	57%	40%			

* Overall Accuracy: 81%, Kappa classification: 0.74

The agriculture category however depicts a completely different picture. There has been a constant declining trend observed in the agricultural lands from 1972 to 2016 (*Table 2*) indicating land cover transformations from agriculture to other classes like

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mining, human settlements and barren lands (*Fig. 2*). The decrease accounts for 28.35% reduction in the total area of agricultural lands. Agriculture covered 46.89% of the study area in 1972 which decreased to 44.08% in 2001 and this is followed by a further decrease in 2016 to 33.59% (*Table 2*).

The fourth category of land cover class that is mining has doubled in the last 44 years. The area statistics shows a noticeable increase of 3.72% in 1972 to 5.16% in 2001 and a further increase to 7.42% in 2016 (*Table 2*). There is an overall increase of 99.48% in the total mining sites. *Fig. 2* shows that although few mining sites have been abandoned and reclaimed, there are new sites within the forested areas which have been leased out to the mining companies. There is also expansion of some existing mining projects into bigger mines and into the forested areas.

The remaining classes of settlement and barren lands show an increasing trend in their areas from 1972 to 2016. Most massive increase is in the human settlement areas which increased from 2.99% in 1972 and 7.88% in 2001 to 10.70 in 2016 with an overall increase of more than 200%. The barren lands also show more than 50% increase in their representation.

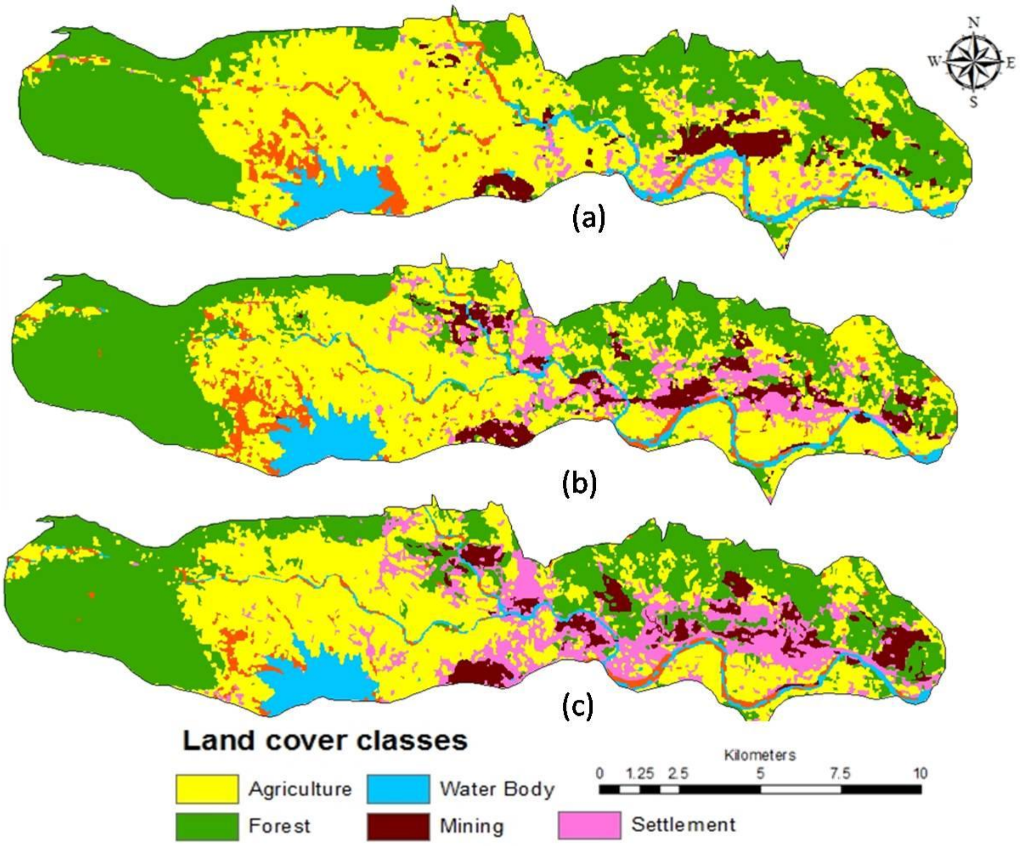

***Figure 2.** Land Cover map 1972 (a), 2001 (b) and 2016 (c)*

Fragmentation dynamics

The fragmentation metrics calculated from the classified images assisted in quantifying the landscape structure and imparted thorough information regarding the configuration changes as well as its impact on the landscape. The analysis revealed that

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there have been major changes in the density, number, size and the distance between the spatially distributed fragments. The analysis at class level also confirmed that different land cover classes exhibited different patterns of changes. The values estimated for 12 class metrics for the East Bokaro coalfields are represented in *Table 4*. The temporal variations in the class metrics for nine selected class metrics are depicted graphically in *Figure 3* for agriculture and forest classes. The detailed dynamics for each category is discussed below.

Table 4. *Dynamics of class metrics for all the land cover classes for 1972, 2001 and 2016*

	Area-Edge				Shape			Aggregation				
Year/Land Cover	NP	PD	LPI	ED	AREA_AM	SHAPE	FRAC	PARA	PAFRAC	ENN	IJI	AI
1972												
Agriculture	123	0.47	29.96	27.88	5103.97	6.80	1.21	62.99	1.42	95.48	89.52	93.62
Forest	121	0.47	18.00	13.53	3270.57	3.92	1.15	43.44	1.33	94.77	36.47	95.77
Water Bodies	22	0.08	2.97	4.56	632.74	4.88	1.18	91.31	1.48	245.15	74.02	91.28
Mining	44	0.17	1.75	3.61	251.34	2.20	1.11	99.67	1.28	189.74	55.31	90.59
Settlement	157	0.61	0.35	7.03	27.71	1.90	1.10	235.37	1.42	250.09	37.91	75.97
Barren Lands	104	0.40	0.60	7.11	59.26	2.24	1.12	174.94	1.45	249.02	45.51	82.36
2001												
Agriculture	303	1.17	23.42	33.41	3338.00	6.51	1.19	79.94	1.39	66.58	88.65	94.56
Forest	152	0.59	19.73	17.78	3393.65	4.24	1.16	59.55	1.37	80.14	48.79	96.05
Water Bodies	32	0.12	3.25	6.13	537.06	3.50	1.16	116.11	1.50	93.27	78.18	92.42
Mining	48	0.19	0.85	6.77	130.77	2.85	1.14	126.96	1.38	278.18	77.81	91.65
Settlement	165	0.64	1.16	12.44	120.95	3.03	1.15	175.45	1.43	124.71	69.24	88.08
Barren Lands	152	0.59	1.51	9.31	151.85	3.49	1.16	232.00	1.50	198.71	54.80	84.21
2016												
Agriculture	284	1.10	22.52	27.82	3556.75	6.05	1.19	78.25	1.39	73.64	79.58	94.42
Forest	127	0.49	18.86	18.13	3202.28	4.63	1.18	57.73	1.41	72.95	64.32	95.97
Water Bodies	41	0.16	3.51	6.30	598.93	3.17	1.15	120.86	1.47	86.86	84.59	91.66
Mining	48	0.19	0.90	6.32	119.47	2.18	1.11	113.22	1.31	227.90	61.08	92.23
Settlement	142	0.55	5.60	19.50	773.38	7.20	1.23	149.19	1.55	92.74	77.97	89.27
Barren Lands	89	0.34	0.41	6.21	38.58	2.47	1.14	241.93	1.55	312.11	69.86	82.81

*Note: Please refer Table 1 for units and abbreviations.

Agriculture

Agriculture is the dominant land cover category in the study area and it shows significant decrease during the entire study period from 1972 to 2016 (*Table 4*). Among the twelve fragmentation metrics displayed in *Table 4* the parameters that are very important to note are the number of patches, patch density and the largest patch index. Increase in the number of patches signifies that an area of land is continuously being broken into smaller pieces or fragments. The agriculture lands show a very steep rise in the number of patches from 1972 to 2001 followed by a slight decrease. Similar trend was observed for the patch density also wherein the patch density increased highly from 1972 to 2001. The large patch index decreased successively from 29.96 in 1972 to 22.52 in 2016 although the shape complexity has decreased in the agriculture lands over time. The Interspersion and Juxtaposition Index signifies that there was a decrease in patch mixing over time for the agriculture as it changed from 89.52 to 79.58.

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Forest

The period from 1972 to 2001 has also seen an increase in the number of patches as well as patch density for the forest lands (*Figure 3*). The largest patch index shows continuous decline for agriculture category from 1972 to 2016, however the forests depict a slightly improved large patch index (*Figure 3*). The shape indices like SHAPE and PARA show that the patch shape complexity of the forested class has also increased over time (*Table 4*) indicating that the patches are becoming more vulnerable to disturbances. On the other hand the forests show an overall increase in the IJI indicating that the forest patches have mixed over time which explains the increase in the large patch index as well as mean patch size. The mean Eucliden Nearest Neighbour distance of forests decreased continously from 94.77 in 1972 to 72.95 in 2016.

Water bodies

The water bodies have undergone very little changes in their total area within the landscape but their fragmentation metrics have changes significantly. There is a steady increase in the number of patches from 22 in 1972 to 41 in 2016 and the patch density from 0.08 in 1972 to 0.16 in 2016 (*Table 4*). The large patch index improved over time with the index being 2.97 in 1972 to 3.51 in 2016 (*Table 4*). This is explained by the increasing interspersion and juxtaposition index that is from 74.02 to 84.59 as well as the very steep fall in the Euclidean nearest neighbor distance from 245.15 to 86.86 since 2016 (*Table 4*). There was also an increase in the edge density of the water bodies from 4.56 in 1972 to 6.30 in 2016. Although, the shape complexity decreased from 4.88 to 3.17 from 1972-2016 the perimeter to area ration increased from 91.31 to 120.86 (*Table 4*).

Mining

Due to growing mining and developmental activities, the number of patches for mining, settlement and barren lands also increased till 2001 (*Table 4*). This can probably be due to the cropping up of new mining sites and settlement points. The year 2001 onwards has seen a decrease in the number of patches as well as patch density for these categories indicating the expansion of mines and merging of smaller settlements into larger settlement groups by clearing the agricultural lands. The mean patch area for mining has decreased over time and the aggregation index has slightly increased since 1972 to the current period.

Settlements

Patch density and number of patches in the settlement category did not change significantly and they improved in 2016 after an initial increase in 2001. The largest patch Index has also increased to 5.60 (2016) as compared to 0.35 in 1972 (*Table 4*). Edge density is a measure of the per unit edge length and the settlement class has undergone a high change in edge density. The edge density increased from 7.03 to 19.50 in 2016.The shape complexity has increased to a great extent for the settlements with the shape index values ranging from 1.90 in 1972 to 7.20 in 2016 (*Table 4*) but the perimeter to area ration decreased considerably. For the aggregation metrics it was found that there was tendency for the patches to mix which is indicated by the IJI and the AI values increasing steadily. There is also a simultaneous decriease observed in the ENN distance between the similar patches.

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Barren lands

For the barren lands and the scrublands, it was observed that though it occupies only 10-18% of landscape area, the number of patches is comparable to the cultivated and the forested lands. The patch density too is the second highest after the settlements. The only important things to note for this category are the mean patch area, PARA and the IJI indices. The perimeter to area ratio increased from 174.94 in 1972 to 241.93 in 2016 while the IJI increased from 45.51 to 69.86 from 1972-2016 (*Table 4*). The mean patch size increased to 151.85 in 2001 but it decreased drastically to 38.58 in 2016.

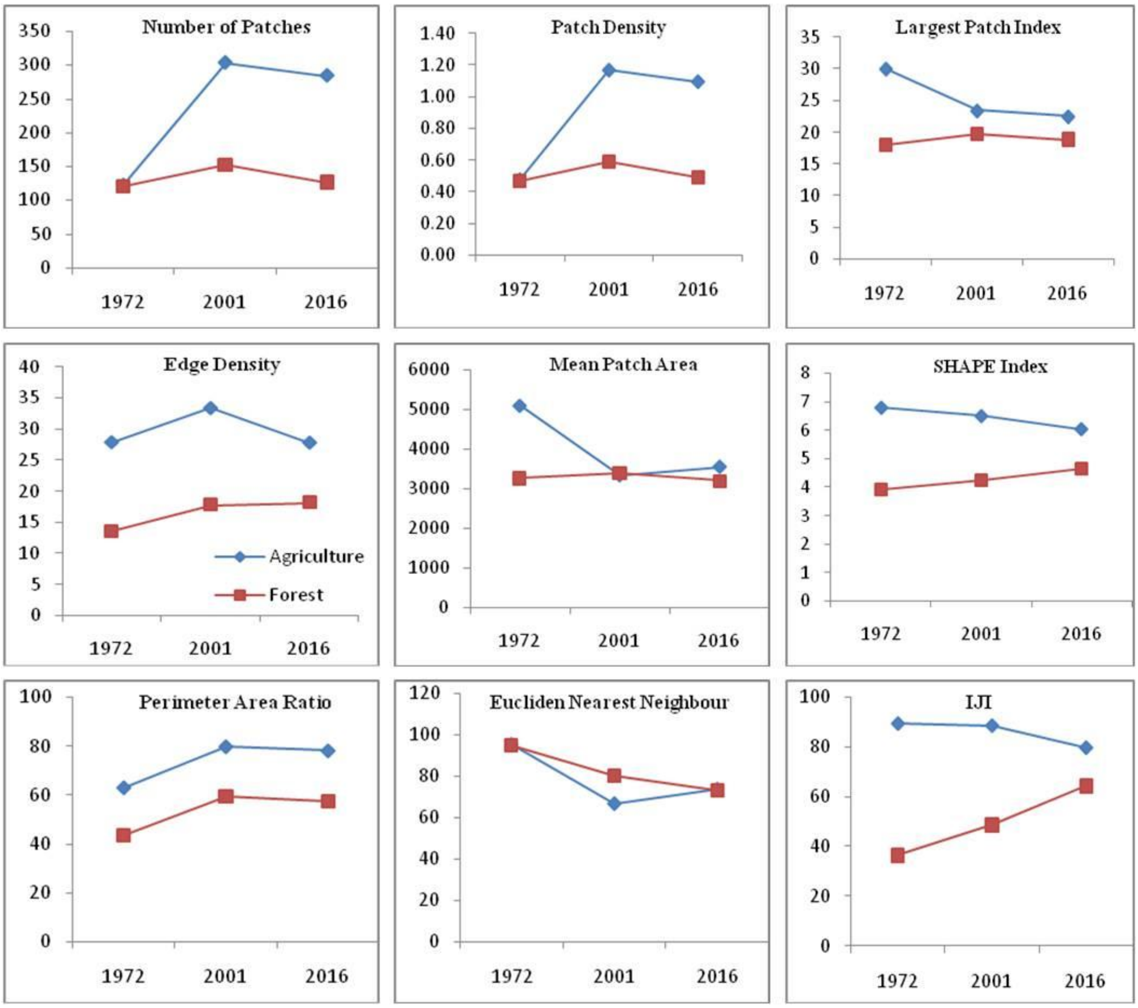

Figure 3. *Temporal changes in the fragmentation metrics of forests and agriculture in the study area*

Discussions

Mining activities extend their ecological footprints beyond the area that is directly impacted. Within the primary impact zone, mining causes complete removal of forest and changes in the composition of any remnant forest while the adjacent areas are the secondary impact zones. The secondary impacts are manifested in the form of land use changes including fragmentation, habitat modifications, wildlife migration and changes in forest types. The developmental activities associated with mining like roads, building,

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and transmission lines lead to forest fragmentation which in turn exposes the interiors of forest to disturbances.

In the current study, the unfolding of the change dynamics of each land cover type, as shown in *Table 2*, *Table 3* and *Figure 3* shows that over the last 44 years the dynamics of evolution are different for the forest and agriculture categories but quite similar for the mining, settlements and barren land categories. Analysis of the trend line revealed that the pattern of land cover changes did not follow a linear pattern and it worked both ways over the 44 year study period. This suggested that the some of the land cover types like forests and water bodies showed increasing and decreasing trends. The forest cover declined at a faster rate from 1992-2001, while the decline rate decreased from 2001-2016. This high decline rate was due to more and more acquisition of forested lands (along with the agricultural lands) by mining projects. However, there is an overall decline in the forest cover but also a substantial increase in the forest cover was observed since 2001. *Figure 2* shows that the increase in forest cover is mostly confined in and around the previously mined and abandoned mines and can be attributed to the vigorous plantation activities being carried out in the reclaimed mine sites and over burden dumps. The subsidiaries of Coal India Limited have carried out extensive tree plantation programmes which include plantations on overburden dumps, plantation around mines, residential colonies and avenue plantations. Coal India planted over 73 millions of plants since 1993-94 covering an estimated land area of over 32000 hectares (CIL). Plantation activities have been integrated into the annual activities of all the existing and new coal subsidiaries.

There was substantial decrease in agricultural land observed in the study area (*Table 2*). This decrease is apparently due to the anthropogenic activities acting in conjunction with the commercial, political and socio-economic factors. The shrinkage of agricultural lands is driven by the diversion of lands for new mining projects, already rugged terrain as well as non profitable agribusiness. This has resulted in shifting the interest of people from conserving the land for agriculture to other more lucrative livelihood options like jobs in mining and allied industries thusultimately ruining the biodiversity. Agriculture and forests have always been the main source of livelihood in the area and some parts are still dependent on agriculture for their livelihoods. The decline in water bodies though not very significant yet the area is suffering from the shortage of availability of clean water for drinking and irrigation purposes.

The area occupied by mining seems to be steadily gaining in appearance from 1972-2016 (*Table 4*). The advent of mining and its increasing representation in the landscape has completely disturbed the ecology which is at high risk of degradation and large scale land use changes. There is a high rate of immigration of people from surrounding regions into the area for livelihood opportunities in the mining sectors. The tremendous mining practices ongoing in the East Bokaro region has led to the evolving new settlements areas concentrating around the mining sites. The settlement areas have increased by more than 250% since 1972 causing a sudden burst in human population and threatening the ecology of the area. The increasing barren lands are an indication of the changing land use practices in the study area.

The framentation outputs depicted the level of landscape configuration changes occurring in East Bokaro. The figures reveal that the different land cover types have undergone varied levels of fragmentation during the study period. The large patch index of agriculture class decreased successively from 1972 to 2016 indicating towards an increasing division of land into other land cover classes. There is observed a high

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increase in the number of patches as well as the patch density from 1972 to 2001 which signals towards high fragmentation rates (*Table 4*). The decreasing intermixing of the patches as indicated by the fall in IJI values show that over the period of time the patches do not show mixing and this might be due to high human interventions.

The increased patch number and density with improved large patch index of the forest class till 2001 implied that the fragmentation of the forests was strengthened during 1972 to 2001 period but decreased from 2001 onwards. The IJI values show that the forest patches have mixed over time. This is quite evident in 2016, where the level of landscape fragmentation as observed for the forest class is not very high. The decreasing ENN index values is due to new plantation areas which can be credited to the vigorous plantation efforts by the mining companies as a part of their reclamation policies. But the SHAPE and PARA indices show that the increasing complex shape of forest patches with a high perimeter to area ratio points towards higher edge influence and decline in the forest interior. Patch shape and patch size interaction can have important implications for some ecological phenomenon. According to Saunders et al. 1991 and Harris 1984, there are large number of evidences to prove that habitat fragmentation may have deleterious effects on species and lead to substantial loss of local and global biodiversity.

Conclusions

In the current study, a multi-temporal approach integrating remote sensing, geographical information system and ground observations was used to assess the spatial changes in various land use categories and the degree of land cover fragmentation. From the analysis, it was concluded that the East Bokaro coalfields is a highly fragile ecosystem which is vulnerable to degradation and drastic land use changes. Agricultural lands registered a drastic decrease in their representation as compared to any other land use category. The plantation efforts have improved the representation of the forests in the landscape but their ecological role still remains threatened. The other land use categories like mining, settlements and barren lands have also increased considerably. The increasing human disturbance driven by coal mining and associated industries has made the forest patches more vulnerable to fragmentation. The study emphasizes that the increasing mining accompanied by rising anthropogenic activities and unsustainable practices are posing a potential threat to the region's local biodiversity. Fragmentation analysis deals with the detailed dynamics of each land cover category (Uuemaa et al., 2013) and this study gives up-to-date information on the level of fragmentation operating in the area. Remote sensing knowledge information coupled with landscape fragmentation can be very advantageous in evaluating the land cover changes and the process of habitat fragmentation occurring at different locations at different periods of time. Thus it is very important to understand such changes in the landscape composition as well as configuration at different temporal scales and their implications so that they can be utilized in appropriate landscape management planning. Such studies augment the requirement of utilizing a landscape level approach rather than patch level approach for policy and management planning. There is a need to manage entire landscapes, not just the components.

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VARIATION OF THE BACTERIAL COMMUNITIES IN THE RHIZOSPHERE OF THREE SPECIES OF THE GENUS *TAGETES* (MARIGOLD) OVER TIME

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Abstract. In the present study, the molecular profiles of rhizospheric bacterial communities of *Tagetes terniflora*, *T. remotiflora* and *T. coronopifolia* were analyzed at different times using soil samples under greenhouse conditions. To this end, samples spanning were obtained at 3, 30, 60 and 90 days. Metagenomic DNA was extracted and the 16S rDNA genes were amplified by PCR-touchdown to reduces the formation of spurious by-products. The amplified fragments were run through Denaturing Gradient Gel Electrophoresis (DGGE) to obtain their molecular profiles. Shannon's index showed a decline of bacterial diversity over time. Also, to visualize spatial proximities by species, a non-metric MDS ordination was conducted, generating a stress value of 0.179. A one way-ANOSIM showed significant differences between *T. terniflora* and *T. coronopifolia* ($R = 0.78$, $p < 0.05$), and between *T. remotiflora* and *T. coronopifolia* ($R = 0.791$, $p < 0.05$). Sequencing of some DGGE profiles bands showed that *Pseudomonas* was present at 3, 30 and 60 days in all species of *Tagetes*, while *Chloroflexus* and *Delftia* were present at 3 and 60 days in *T. remotiflora* and *T. coronopifolia*, respectively. The study demonstrated that the beneficial populations were positively selected and sample time and species affect the dynamic succession in the rhizobacteria communities.
Keywords: *nMDS, DGGE, 16S rRNA, V3 hypervariable region, Bray-Curtis similarity index*

Introduction

The term microbial diversity is used to explain the composition, complexity and structure of microbial communities in natural systems. In ecosystems, such as soil and rhizosphere, diverse microbial consortia play an important role in biogeochemical cycles, decomposition and mineralization of organic matter, as well as the formation and maintenance of edaphic structure that confer soil quality (Johnson et al., 2003; Kirk et al., 2004; Ranjard et al., 2010). Besides, the microbial communities influence the nutrition and health of plants through the processes of mineral solubilization, hormone production, and nitrogen fixation at the same time that also antagonizing pathogenic microorganisms (Petersen et al., 1996; Pinton et al., 2001; Prosser, 2002; Nannipieri et al., 2003; Saharan et al., 2011).

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In rhizospheric environments, which are considered a complex example of ecological equilibrium between microbiome and plant roots, most of the environmental changes that occur are attributed to organic substances exuded by the roots, which constitute a nutrient source for microorganisms (Hardoim et al., 2015; Chiarini et al., 1994; Rovira, 1965). However, the composition of rhizospheric exudates is affected by the stage of plant development (Hamlen et al., 1972), which can lead to the selection of specific bacterial genotypes (Picard et al., 2000) that may influence changes in the structure, patterns and activities of rhizobacterial communities (Di Cello et al., 1997; Hamlen et al., 1972; Chen et al., 2014) as has been documented in several microbial communities through an habitat filtering or by interspecific competition (Pontarp et al., 2012; Crits-Cristoph et al., 2013; Zelezniak et al., 2015; Centeno et al., 2016).

Actually, there is a growing interest in the allelopathic properties of plants of the genus *Tagetes* due that, the alternated cultivation of these plants with economically-important crops such as tomato, potato, mulberry, strawberry, soybean, pineapple, cabbage, cauliflower, lettuce, taro and carrot had been successful in the control of plagues (Hooks et al., 2010) mainly by the allelochemical substances produced in flowers, leaves, stems and roots (Weidenhamer et al., 2009). Experimental evidence suggests that certain allelochemical substances, such as α-terthienyles, produce oxygen-free radicals that can significantly reduce populations of root knot nematodes, insects, fungi, bacteria, and some viruses (Tereschuk et al., 1997; Nivsarkar et al., 2001; Tomova et al., 2005). Nevertheless, little is known about the changes in the composition of bacterial communities in the rhizosphere over time. Hence, the aim of this study was to analyze the antagonistic effect of three species of the genus *Tagetes* on changes in rhizosphere bacterial diversity and community structure at different growth stages. To this end, molecular profiles of the V3 hypervariable region of the 16S rRNA gene were obtained using the Denaturalizing Gradient Gel Electrophoresis technique (DGGE) approach.

Materials and Methods

Soil samples

Soil samples were collected in La Vega de Metztitlán region in the state of Hidalgo, Mexico, a zone considered the main agricultural area in the Barranca de Metztitlán Biosphere Reserve located at 20° 42’ 12”; 20° 28’ 04” N and 98° 53’ 20”; 98° 40’ 21” W, at a mean altitude of 1,270 m (4,167 ft.) above sea level (Guzmán et al., 2008). Ten samples were taken randomly in a 5-hectare area (12.4 acres) of agricultural fields planted with chili peppers near the locality of Tres Cruces. First, the upper layer of soil was removed (≈5 cm), then, approximately 50 kg of soil were collected, placed in sterile polyethylene bags, and sealed with elastic bands. After it, in the laboratory, the ten samples were mixed to yield one composite sample, which placed in polypropylene bags in 2 kg quantities and autoclaved for 45 minutes at 121°C, this process was repeated three times with 24 hours of difference.

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Tagetes crop

To establish the crop of *Tagetes* plants under greenhouse conditions, seeds of three species were used: *T. terniflora*, *T. remotiflora* and *T. coronopifolia*, all provided by Dr. Miguel Ángel Serrato Cruz of the Department of Plant Science at the Universidad Autónoma de Chapingo, Mexico. Planting the three *Tagetes* species was conducted as follows: 10-12 seeds were germinated in glass Petri dishes using filter paper moistened with distilled water as support. To maintain humidity, the Petri dishes were placed on a plastic tray (20 × 15 × 5 cm) and covered with cellophane plastic that contained moist paper towels. Under these conditions, the seeds were exposed to solar light and environmental temperature until germination. The distilled water was changed every 24 h by adding a fresh volume of 10 mL to prevent the seeds from rotting. At 8-14 days after germination, 10 seedlings of each species were transplanted into plastic boxes (72 × 42 × 66 cm) containing sterile composite soil sample to ¾ of their capacity. The seedlings were separated by 15 cm to obtain better development. Finally, a control with no plants was established.

Samples of rhizospheric soil

To evaluate changes in the composition of the bacterial community, sampling of the rhizospheric soils was performed at 3, 30, 60 and 90 days after transplanting the seedlings. For each specie and stage, was collected and chosen at random, a unique whole plant with adhering soil. The rhizosphere soil was obtained removing the loose soil by careful shaking, after which tightly-adhering soil was sampled from the root surfaces of each plant, we obtained three replicates of approximately 1.5 g of rhizospheric soil, which was placed in 2-ml polypropylene tubes and stored at 4°C.

The physicochemical analysis of the rhizospheric soil collected in La Vega (Metztitlán) was conducted using the Motte Turf Lab Deluxe kit (model TL-2®, Cáceres, Spain) following the protocol provided by the supplier. Analysis consisted in determining nitrogen (N), total phosphorus (P_{tot}), potassium (K) and iron (Fe) content, pH, and soil texture. Determination of the soil texture type was based on data obtained for the percentages of clay, sand and loam, using a triangle of soil texture in accordance with the Official Mexican Norm NOM-021-RECNAT-2000.

Extraction of nucleic acids

Extraction of metagenomic DNA from the rhizospheric soil and controls was carried out using the protocol described by Cullen and Hirsch (1998), the procedure basically was lysis of indigenous soil microorganisms using alkaline-SDS buffer in a bead-beater. Purification of the extracted metagenomic DNA was performed with the commercial Zymo Research ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research Corp, Irvine, CA, U.S.A.), following the manufacturer's instructions. The quality of the DNA was observed in agarose gels at 1% with TAE 1X regulator.

DGGE-PCR

DGGE-PCR amplification of the V3 hypervariable region of the bacterial 16S rRNA gene (193 bp) from the extracted and purified rhizospheric DNA was conducted with the

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universal oligonucleotides *P3* (5'- CCT ACG GGA GGC AGC AG-3'), and *P2* (5'-ATT ACC GCG GCT GG-3'). A GC clamp of 40 nucleotides (5'-CGC CCG CGC GCG GGC GGG GCG GGG GCA CGG GGG G-3') was added to the forward oligonucleotide *P3* at position 5' (Muyzer et al., 1993). Touchdown PCR was performed in a total reaction volume of 100 µl. The concentrations of each component of the reaction mix were 5 ng of DNA, 1X PCR buffer (20 mM Tris-HCl pH 8.0 and 50 mM KCl), 2.5 mM of $MgCl_2$, 250.0 mM of each dNTP, 0.2 mM of each initiator, 0.25 OR/µl of *Taq* DNA polymerase (Invitrogen®) and 250 ng/µl of BSA (Amresco®). The amplification conditions of Touchdown PCR were conducted with an initial cycle at 95°C for 10 min. Next, the alignment temperature was reduced from 65°C to 50°C in intervals of 1°C during 15 cycles. An additional 20 cycles were run at an alignment temperature of 50°C. Each cycle involved a denaturalizing temperature of 94°C for 1 min and an extension temperature of 72°C for 90 s, followed by a final extension at 72°C for 5 min.

Denaturalizing Gradient Gel Electrophoresis (DGGE) was performed following the methodology described by Muyzer et al., (1993). Polyacrylamide gels at 7% were prepared (16 × 16 cm) with a chemical denaturalizing gradient of 0-80% utilizing a urea solution at 5.6 M and formamide at 32% (Sigma-Aldrich, St. Louis, MO, U.S.A.). 20 µl of the Touchdown PCR product were added to each well of the gel, and then run them in an electrophoresis chamber (DGGE-2001, CBS Scientific, San Diego, CA, U.S.A.) that contained TAE 0.5X regulator. Electrophoresis was pre-run for 20 min at 200 V and then for 15 h at 60°C and 70 V. Visualization of the DNA bands was carried out using silver stain from the commercial PageSilver™ Silver Staining Kit (Fermentas, Glen Burnie, Maryland, U.S.A.).

DNA sequencing and phylogenetic reconstruction

The amplified and purified fragments from the V3 region of the 16S rDNA gene were sequenced in both senses using DGGE-PCR initiators (but without the GC clamp) in an automatic 3130 Applied Biosystems® Genetic Analyzer sequencer with 4 capillaries (Foster City, CA, U.S.A.) at the Department of Seed Production at the Colegio de Posgraduados Chapingo. Once the DNA sequences were obtained, they were compared using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to identify similar sequences in the database of nucleotide deposited at the National Center for Biotechnology Information (NCBI). The sequences were edited manually with the help of the BioEdit program V.7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and aligned using the program Clustal X V.2.1 (Thompson et al., 1997). The phylogenetic relationships of the sequences were determined by maximum likelihood analysis (ML) using the PhyML program (http://atgc.lirmm.fr/phyml/) (Guindon et al., 2010). The optimal model of the evolution of our sequence data was obtained using the Akaike Information Criterion (AIC) with Modeltest program V.3.7 (Posada and Crandall, 1998). The support values for each node were estimated using 5,000 bootstrap replicates.

Statistical and DGGE analyses

DGGE gel banding patterns were examined using the 1D gel electrophoresis image analysis software GelAnalyzer (http://www.gelanalyzer.com/index.html). Each band was coded as present (1) or absent (0), and described by its position in the banding profiles.

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Shannon's diversity indexes were estimated for each profile. Also, binary data were transformed mathematically for normalization by the equation x'= log 10 (x + 1) before applying the Bray-Curtis index (Legendre and Legendre, 2012).

The PCR-DGGE-generated banding patterns of the soil samples obtained from the rhizosphere of the three *Tagetes* species and the control at different times were analyzed using a clustering algorithm from the Bray-Curtis index. Additionally, PERMANOVA (Permutational Multivariate Analysis of Variance Using Distance Matrices) with 5000 permutations was performed to test for significant differences between species and development stage in the bacterial community of the rhizosphere. Cluster analysis and dendrogram generation were carried out using PAST software package (PAleontological STatistics) v.3.0. (Hammer et al., 2001).

To state the changes in the bacterial community composition, a one-way ANOSIM analysis with 999 permutations was performed using *Tagetes* species and sampling time as the explanatory variables. This analysis allowed us to evaluate the null hypothesis, H_0, which indicated that there were no significant differences in the structure of the bacterial communities of the three species of *Tagetes*. For each ANOSIM test, we calculated separately the statistical Global R at a significance level of $p < 0.05$ to reject the null hypothesis. Also, species turnover bacterial communities were estimated using the Whittaker Beta Diversity index.

Also, a non-metric multidimensional scaling analysis (nMDS) was performed with 999 bootstraps to avoid minimal local (Legendre and Legendre, 2012). Both multivariate (ANOSIM and nMDS) and beta-diversity analyses were performed using the Bray-Curtis similarity index with the aforementioned PAST software package.

Results

Soil analysis

The physicochemical analysis of the composite soil collected in La Vega de Metztitlán, and used to grow the *Tagetes* plants, was found to contain the following quantities: NO_3-N = 5.60 g/m^2; P_{tot} = 0.56 g/m^2; K = 17.933 g/m^2; and Fe = 1.5 ppm; while pH was 7.0. The analysis of soil texture demonstrated that it was made up of 53.3% sand, 16.7% loam, and 30.0% clay, and so may be considered of the sandy clay loam type.

DGGE patterns and bacterial diversity

Nine samples obtained from the rhizospheres soils of the three *Tagetes* species at different times, plus three control samples, were subjected to electrophoresis in two acrylamide gels under denaturing conditions. Analysis of the gels using GelAnalyzer software (V. 2010a, freeware; Istvan Lazar) showed profiles that were both complex and diverse and that together made it possible to identify a total of 101 bands. The relative intensity or crude volume of each band was calculated after staining with $AgNO_3$, which produced values in the range of 0.43-7.8%. The average number of bands per sample was 37.1. Specifically, for the four sampling times in *T. terniflora*, the average number of bands was 43.6, while the averages calculated for *T. remotiflora* and *T. coronopifolia* were 35.4 and 32.4 bands, respectively. The number of bands in the molecular profiles for each time

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and species decreased as time increased. Thus, the analysis of triplicate gels showed that at 3 days *T. terniflora* presented, on average, 50 ± 4.3, at 30 days 52 ± 1.7, at 60 days 39 ± 4.7, and at 90 days 35 ± 6.0 bands; *T. remotiflora* at 3 days 42 ± 4.3, at 30 days 31 ± 2.7, at 60 days 34 ± 6.0, and at 90 days 36 ± 6.0 bands; *T. coronopifolia* at 3 days 39 ± 4.0, at 30 days 34 ± 4.0, at 60 days 29 ± 4.3, and at 90 days 29 ± 5.0 bands; and controls at 3 days ± 2.4 33, at 30 days 32 ± 2.4, at 60 days 29 ± 1.4, and at 90 days 23 ± 1.4 bands.

Due to the allelopathic properties of the roots of *Tagetes terniflora*, *T. remotiflora* and *T. coronopifolia*, an analysis of variation of rhizosphere bacterial communities in soil was carried out using the molecular profiles generated by DGGE at 3, 30, 60 and 90 days of growth (*Figure 1A*). The DGGE profiles were reproducible in triplicate, regardless of the DNA extraction process. Profiles were analyzed utilizing a criterion of coincident bands, which allows the construction of dendrograms and calculations of diversity indexes. The dendrogram shown in *Figure 1B* represents the similarity of the relationships among the molecular profiles obtained from the samples of rhizospheric soils at the different times of development for the three species of *Tagetes*. This dendrogram presents a cophenetic correlation coefficient of 0.9054, using a Bray-Curtis similarity measure and 5000 replicates. This analysis made it possible to recover two main clusters, one that included the control and *T. coronipifolia*, and a second with *T. terniflora and T. remotiflora*. In the main cluster, all samples from the same treatment were recovered jointly. In the control, the most similar communities were found between the samples collected at time 3 and 30 (similarity above 96%), while the sample collected at time 90 showed the greatest difference, with a similarity of just 80%. The similarity values calculated for the *T. coronipifolia* samples collected at time 3 and 30 and times 60 and 90 were quite close to each other (≈60%). For *T. terniflora*, the most similar communities were found between times 3 and 30 (≈70%), while the sample taken at time 90 differed most markedly (≈46%). Finally, for *T. remotiflora*, the most similar communities were found between times 30 and 60 (≈56%), and the most different sample was the initial community sampled at time 3 (≈46%).

Whittaker beta diversity (β_w) indexes were estimated to detect changes in bacterial composition among species and over time. All estimated values were above zero, even in the control sample over time. Beta diversity values ranged from $\beta_w = 0.014$ in the control sample between days 3 and 30 to $\beta_w = 0.827$ between the control sample at day 90 and *T. remotiflora* at day 30. The low beta diversity values correspond to the control treatment at the different times.

These Whittaker global beta diversity values show that *T. coronopifolia* had the highest rate of species turnover at $G\beta_w = 1.100$, while *T. terniflora* and *T. remotiflora* showed values of $G\beta_w = 0.847$ and $G\beta_w = 1.040$, respectively. The Mantel test using 9999 permutations suggests that regardless of the changes in bacterial species during the sampling times for each species, the relations between beta diversity and time were statistically-distinct from zero ($r = 0.0049$, $p = 0.4312$).

As a means of measuring alpha diversity, we estimated Shannon's diversity indexes for the electrophoretic profiles generated by the rhizosphere of *T. terniflora*, *T. remotiflora* and *T. coronopifolia* (*Table 1*). The indexes of biodiversity estimated for *T. terniflora* presented a value of 3,573 on day 3, though this decreased at 30 and 60 days to values of 3,345 and 3,445, respectively. However, at day 90 the value increased to 3,468. In the cases of *T.*

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remotiflora and *T. coronopifolia*, observations discerned a downward tendency in the estimated diversity indexes. The boxplot graphs in *Figure 2* show the diversity of the molecular bands in the DGGE profiles. Of the three species of *Tagetes* used, the greatest band diversity was observed in *T. coronopifolia*.

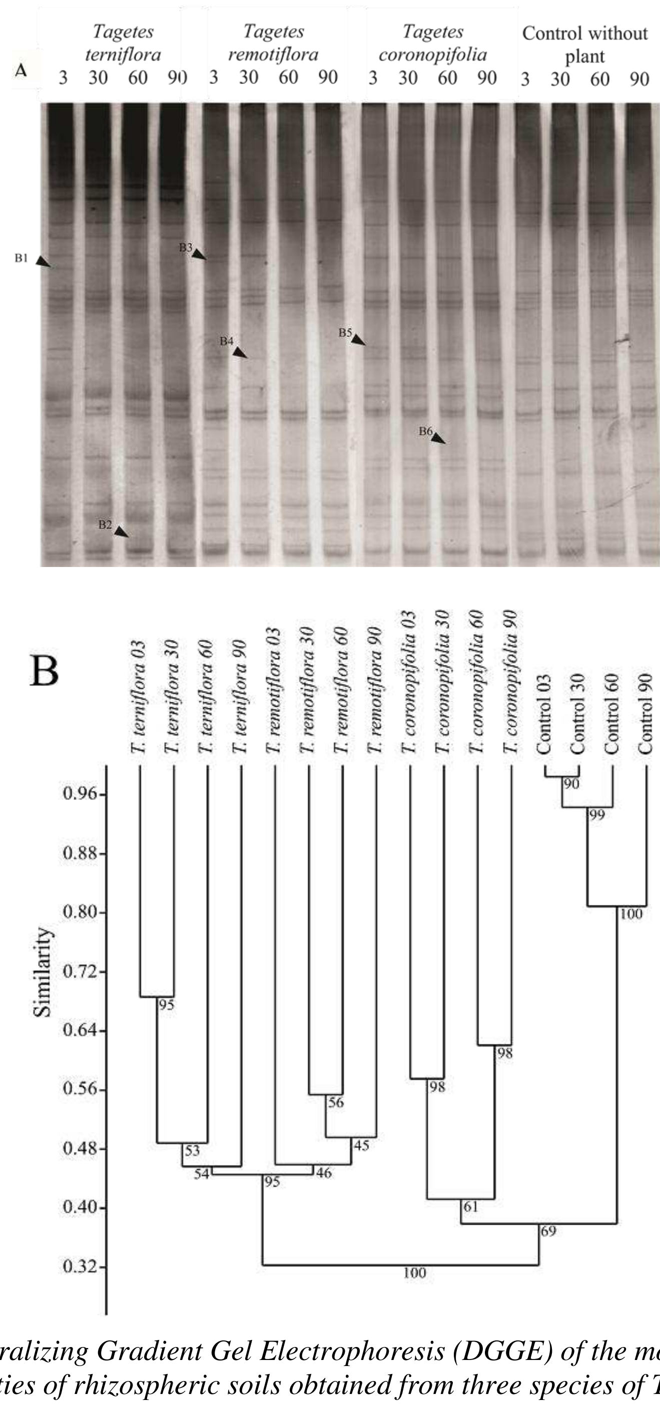

Figure 1. A. *Denaturalizing Gradient Gel Electrophoresis (DGGE) of the molecular profiles of the bacterial communities of rhizospheric soils obtained from three species of Tagetes and controls;* ***B.*** *Dendrogram constructed on the basis of the DGGE molecular profiles, utilizing Jaccard's similarity coefficient, grouped by Neighbor-Joining*

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Table 1. *Shannon's diversity indexes*[1]

Sample	Shannon's Diversity Index[1]			
	3 days	**30 days**	**60 days**	**90 days**
Rhizospheric soil from *Tagetes remotiflora*	3.738 (0.043)[2]	3.434 (0.043)	3.526 (0.061)	3.584 (0.053)
Rhizospheric soil from *Tagetes terniflora*	3.912 (0.043)	3.951 (0.017)	3.664 (0.0)	3.555 (0.0)
Rhizospheric soil from *Tagetes coronopifolia*	3.664 (0.031)	3.526 (0.041)	3.367 (0.0)	3.367 (0.050)
Non-rhizospheric soil control	3.497 (0.0)	3.466 (0.0)	3.367 (0.0)	3.135 (0.0)

[1]Biodiversity indexes for repetitions of samples (n=3) estimated using Shannon's Diversity Index. $H = -\Sigma p_i \ln p_i$. Means ($\bar{x}$) and standard deviations (SD) are shown together with the diversity values.

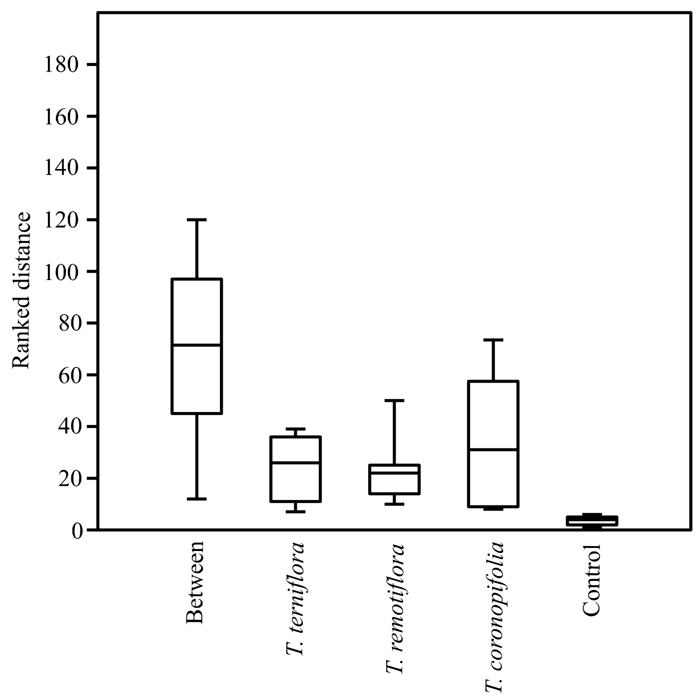

Figure 2. *Boxplot of the mean Bray-Curtis distance measures of DGGE profiles obtained from rhizosphere soil samples of three species of the genus Tagetes*

nMDS analysis of DGGE banding patterns

Analysis with a non-metric MDS ordination in two dimensions and Bray-Curtis similarity measure resulted in a stress value of 0.232. However, adjustment for three dimensions produced a stress value of 0.179, which allowed a better interpretation of the nMDS plot (*Figure 3*). This plot showed the separation of three groups of bacterial populations. The first was located at the top right and corresponds to the control samples.

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The second group was situated on the bottom right and includes the samples taken at 3, 30, 60 and 90 days of the species *Tagetes coronopifolia*. Finally, the overlapping groups of *Tagetes terniflora* and *T. remotiflora* were localized to the left of the graph. These results show that the bacterial communities of the control and three species of *Tagetes* differ clearly, as does *T. coronopifolia* with respect to the other two species.

Analysis of similarities (ANOSIM)

The one-way analysis of similarities (ANOSIM) showed significant differences between *T. terniflora* and *T. coronopifolia* (1-way ANOSIM R = 0.78, p <0.05), between *T. remotiflora* and *T. coronopifolia* (1-way ANOSIM R = 0.79, p <0.05), and between each species of *Tagetes* and the control (*Table 2, Figure 2*). The value of the one-way Global R for the three species of *Tagetes* was $R_{Global} = 0.83$ at *p* = <0.05, which allowed us to reject the null hypothesis and demonstrate that the differences among species were significant. On the other hand, the Global R for the factor time was R_{Global} = - 0.028, and p => 0.05, so we were able to accept the null hypothesis of no significant differences with respect to sampling times. The PERMANOVA test showed a significant effect of the *Tagetes species* on the structure of the bacterial community (***F*** = 5.28; ***p*** < 0.01). Significantly, the greatest differences were found between *T. cornopifolia* and *T. terniflora* with *p* = 0.32; *T. cornopifolia* and *T. remotiflora* with *p* = 0.026; and *T. coronopifolia* and the control with *p* = 0.029. No significant differences were found with respect to time (*F* = 0.67, *p* = 0.86).

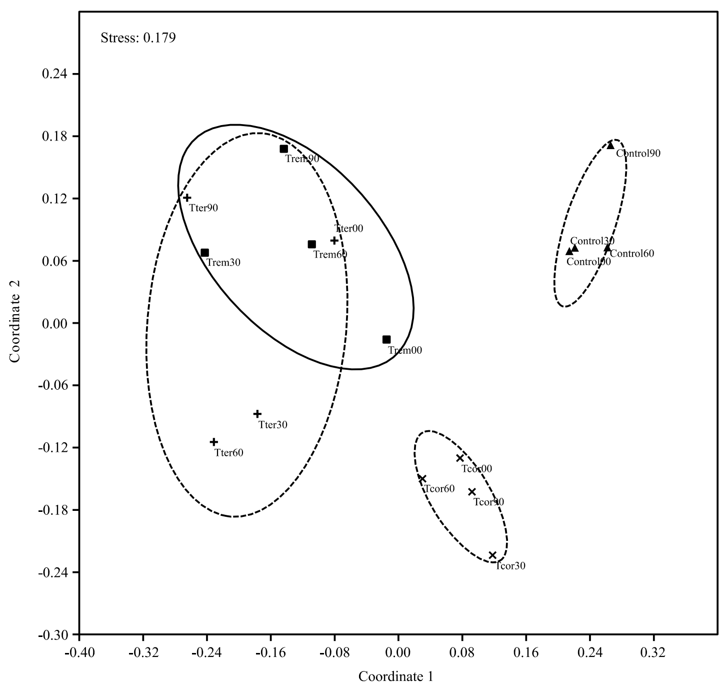

Figure 3. *Two-dimensional plots of nMDS analyses from DGGE patterns to compare differences in bacterial communities taken from rhizosphere soil samples of three species of the genus Tagetes*

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Phylogenetic analysis

Six bands from the DGGE profiles of rhizobacteria communities of *T. terniflora*, *T. remotiflora* and *T. coronopifolia* were selected for molecular identification. The criterion utilized in the selection of the bands was basically their atypical or discontinuous presence or absence at the different sampling times. Band B1 was present at 3 and 30 days, absent at 60 days, and reappeared at 90 days. Band B2 was absent at 3, 30 and 90 days, and only present at 60 days. Band B3 was present at 3 days but absent from the later samples taken at 30, 60 and 90 days. Band B4 was present on days 30 and 60, but absent at 3 and 90 days. Band B5 was present at 3 and 30 days, but absent at 60 and 90 days. Finally, Band B6 was absent at 3 and 30 days, but present at 60 and 90 days (*Figure 1A*).

Figure 4 shows a phylogenetic tree constructed on the basis of the band sequences obtained from the DGGE molecular profiles of the rhizobacteria communities from the soil samples of *T. terniflora*, *T. remotiflora* and *T. coronopifolia*. The phylogenetic analysis shows the relations of bands B1, B2, B4 and B5 to the group of the genus *Pseudomonas*; of band B3 to non-cultured bacteria; and of band B6 to microorganisms of the genus *Delftia*.

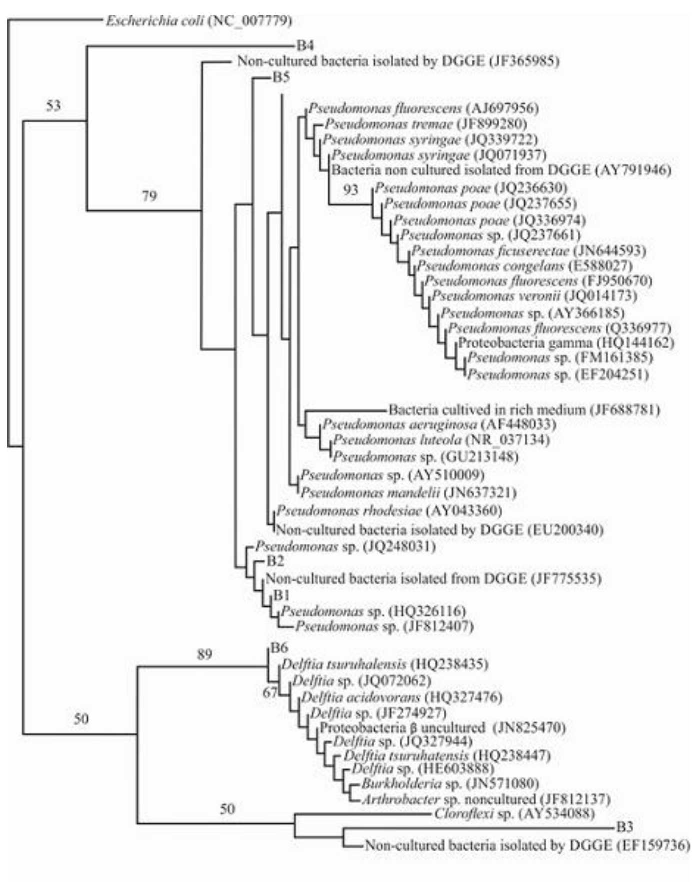

***Figure 4.** Phylogenetic tree constructed on the basis of the sequences from hypervariable region V5 of the 16S rDNA of the bands of interest selected from the molecular profiles of the rhizospheric bacteria in T. terniflora (B1 and B2), T. coronopifolia (B3 and B4) and T. remotiflora (B5 and B6), generated by DGGE*

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Table 2. *ANOSIM statistics of Bray-Curtis similarity measures (R) from the rhizospheres of three species of the genus Tagetes and control*

Rhizosphere	Statistical R	*p* value
T. terniflora vs. *T. remotiflora*	0.38	0.086
T. terniflora vs. *T. coronopifolia*	0.78	0.026
T. remotiflora vs. *T. coronopifolia*	0.79	0.027
T. terniflora vs. Control	0.98	0.026
T. remotiflora vs. Control	1.00	0.031
T. coronopifolia vs. Control	0.79	0.027

Discussion

The nature of the changes in structure and diversity of soil bacterial communities that result from the interactive effects of different plant species is a little-studied issue in Microbial Ecology. The use of techniques based on the Polymerase Chain Reaction (PCR) of metagenomic DNA coupled with Denaturalizing Gradient Gel Electrophoresis (DGGE) allow us to analyze the relative abundance of the populations of dominant bacteria and compare the successions or changes in the structure of microbial communities in environmental samples (Muyzer and Smalla, 1998; Brons and van Elsas, 2008; Rodríguez-Lanetty et al., 2013; Chen et al., 2014). Although the usefulness of these techniques is limited by the number of DNA bands and the complexity of the profiles obtained (Nannipieri et al., 2003; Heuer et al., 2001; McCaig et al., 2001; Curtis et al., 2002), when employed over time, these DNA band profiles can help improve our understanding of the succession of microbial soil communities.

This study analyzed the effect of three species of *Tagetes* on the structure of bacterial communities in samples from rhizosphere at different times. These plants are recognized for their allelopathic action because their roots naturally exude components denominated α-terthienyles (Meissner et al., 2013). The results presented here suggest a heterogeneity in the diversity of bacterial communities in the rhizosphere related with plant species. In spite of we failed to detect a selective sweep that leads to a drastic reduction in the alpha diversity estimated through Shannon index we were able to find a drastic change in the bacterial composition trough time.

In relation to the above mentioned results, several earlier studies have suggested a selective effect of microbial populations in plant rhizospheres driven by edaphological characteristics of the soil (Duineveld et al., 1998; Guong et al., 2012) an effect that is highly-specific and reproducible in several vegetable species (Kumar et al., 2016). Nevertheless, recent works have shown that changes in the microbial communities of the rhizosphere could be related with habitat filtering or intraspecific competition. For example, Shi et al. (2015) analyze the changes in microbial rhizosphere communities of the annual grass *Avena fatua* in two growing sessions and found that in spite of the different starting communities in both seasons, the successional patterns were similar and the final communities were very similar. On the other hand, Yuan et al. (2016) analyzed the rhizosphere of the seepweed *Suaeda salsa* and found that both phylogenetic clustering (abiotic factors) and overdispersion (biotic factors) are involved in the high tolerance to salinity in this plant species.

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The genus *Tagetes* has been characterized as exuding allelopathic compounds of the thiophenes type which have a nematocide function that has been demonstrated in both field and laboratory (Riga et al., 2005). Once isolated and purified, these allelochemical compounds have been used *in vitro*, where they have shown antiviral, antibacterial, antifungal and insecticide properties as well. Although many tests have demonstrated their allelochemical action, by determining the values of Shannon's diversity indexes obtained from sterilized agricultural soil (*Table 1*), the results of the present study demonstrate a possible selective, species-specific effect of plant rhizospheres on the microbial populations in the soil that develops through exudation during their developmental period as has been proposed in other plant species (Singh and Mukerji 2006; Chaparro et al., 2013).

Topp et al., (1998) analyzed the effect of the roots of *Tagetes* sp. on microbial communities in the soil. Their study considered the suppressor effect of the thiophenes, which are heterocyclic molecules with sulfur atoms that by activating α-terthienyl produce oxygen-free radicals. In this perspective, if the roots of *Tagetes* sp. release a biocide that is activated in the soil, then the microbial populations of the rhizosphere of *Tagetes* sp. should be substantially perturbed. In an effort to demonstrate this, those researchers took measurements of the size of microbial populations and their activity in soils from fields and greenhouses where *Tagetes* sp had been cultivated, and compared them to soils from uncultivated yards (with no vegetation) and fields where rye had been planted (*Secale cereale* L.). The results obtained using extraction-fumigation methods to measure the microbial biomass (MB), epifluorescence microscopy (5-[4,6-dichlorotriazine-2-γl] aminofluorescein) to quantify total bacteria, and heterotrophic bacterial plate counts with different media, the most probable number for the count of nitrifying bacteria demonstrated that the size of microbial populations and their activity in the diverse soils had no significant differences (Topp et al., 1998). Similar results have been found with other methods, such as marking the rye residues with ^{14}C and adding them to soils with the different treatments revealed mineralization activities that were slightly faster in the soil cultivated with rye than in the other treatments. In addition, measurements of the mortality rates of bacterial cells of *Escherichia coli* and *Rhodococcus* TE1 introduced into the soils cultivated with *Tagetes* sp. and the other treatments indicated that there was no accumulation of the biocides. These findings allowed the authors to conclude that the allelochemical exudates released by *Tagetes* sp. did not cause a general decline in the number of microorganisms in the soils and, possibly, that the control of nematodes by this plant might proceed through some other mechanism, and is not due to the release of a biocide into the soil. Analyses of the results of the BoxPlot graph (*Figure 2*), the DGGE gel (*Figure 3A*), and the dendrogram (*Figure 3B*) confirm the phenomenon of a specific, heterogeneous selection of bacterial populations by the species of the plant *Tagetes*.

The phylogenetic tree reveals the presence of three principle groups: the first made up of bacteria of the genus *Delftia* (β-*proteobacteria*), the second of bacteria of the genus *Pseudomonas* (γ-*proteobacteria*), and the third a non-cultured bacterium (*Clorofexi*). Shi et al. (2013) detected significant increases in the relative abundances of rhizobacterias in classes α-*proteobacteria*, β-*proteobacteria*, and γ-*proteobacteria*, which are well known rhizosphere colonizers and have generally been characterized as fast-growing r-strategists, which respond positively to low-molecular-weight substrates abundant in plant root exudates.

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The population succession obtained using DGGE demonstrated that bands B1 (3 days, *T.* t*erniflora*), B2 (60 days, *T. terniflora*), B4 (30 days, *T. remotiflora*) and B5 (60 days, *T. coronopifolia*) were variably present at the different times analyzed (*Figure 3A*), while the phylogenetic analysis indicated that these sequenced bands are found in the group of bacteria belonging to the genus *Pseudomonas*. BLAST analysis also demonstrated that all the sequences related to the genus *Pseudomonas* had high similarities to species of *Pseudomonas* isolated from rhizospheric and other environments.

Some species of the genus *Pseudomonas* are often described as plant-growth promoting rhizobacteria (PGPR), due to the fact that they interact with the roots of plants to produce antibiotics, phytohormones, HCN, and siderophores that promote development and protect them from pathogenic microorganisms (Upadhyay and Srivastava, 2010; Subramanian and Saytan, 2014). In this way, together with the use of such techniques as electronic microscopy and genetically-marked strains, studies have shown that the mutual relations entailed in the spatial distribution of the genus *Pseudomonas* are not uniform in the roots of plants, because they prefer to inhabit regions characterized by a higher release of exudation, such as the unions between the epidermal cells of the root, indented parts of the epidermis, or lateral sites on the roots (Chin-A-Woeng et al., 1997; Fukui et al., 1994). On the other hand, observations have also shown that other bacteria –such as *Rhizobium*– prefer the tips of root hairs (Smit et al., 1986; Smit et al., 1987), presumably due to the presence of specific receptors (Swart et al., 1994).

Specific studies of the genus *Pseudomonas* have demonstrated their isolation at high frequencies from soils that suppress black rot in the roots of tobacco plants (*Thielaviopsis basicola*) and most of the fungal diseases that affect wheat (*Gaeumannomyces graminis var. Tritici*). This suppression phenomenon in soils is now well-characterized, and there is strong evidence to indicate that it is the result of the release of the antifungal metabolite 2, 4-diacetilflouroglucinol (PHL) (Walsh et al., 2001). Maize is another crop whose roots promote the colonization of antagonistic groups of *Pseudomonas*, and microbiological quantification has shown that a high proportion (~15%) of the strains of *Pseudomonas* isolated from the rhizoplane contain genes for the biosynthesis of PHL, while non-rhizospheric soils present low levels of this gene (<0.65%) (Picard et al., 2000).

However, in the case of antagonistic plants of the genus *Tagetes*, mutual relationships seem to be more complex, since previous studies have reported the presence and exudation of essential oils with distinct compositions that were common to different species of this genus (Lawrence, 1985; Héthélyi et al., 1986; Marotti et al., 2004). The most remarkable characteristic of the metabolites produced by the genus *Tagetes* is that they have toxic activity on various microorganisms that are pathogenic to plants, animals (Eguaras et al., 2005) and humans (Cestari et al., 2004). Therefore, this genus is a potential option for use in agriculture and medicine as a natural supplier of biocides, biorepellents, or biostatic effects (Díaz-Cedillo and Serrato-Cruz, 2011).

Band B3 is similar to the group of non-cultured bacteria of the genus *Clorofexi*. A recent study (Krzmarzick et al., 2012) demonstrated that in terrestrial environments this genus naturally utilizes organochlorine compounds as final electron-accepters. It is a well-known fact that plants, marine organisms, insects, bacteria, fungi and mammals all naturally produce organochlorine compounds (Öberg, 2002; Öberg and Sandén 2005; Krzmarzick et al., 2012), and this has helped us to better understand that in terrestrial environments the

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transformation of chlorides into organochlorine compounds occurs through the activity of the enzyme chloroperoxidase that results in levels of organochlorine compounds that tend to be higher than those of chloride on the soil surface. As soil depth increases, changes in the speciation of chlorine take place, predominantly from organic-to-inorganic. This suggests that the organochlorines that exist naturally in the organic matter of the soil may be subjected to such biogeochemical processes of chlorination (Krzmarzick et al., 2012).

In the case of plants of the genus *Tagetes*, some 126 secondary metabolites have been identified with diverse carbon skeletons, including the thiophenes, of which 5-(but-l-chloro-2-ol-3-ynyl)-2,2′-bithienyl is characterized as containing a chlorine atom in its R2 (Xu et al., 2012). However, the presence of Band B3 at 3 days after transplanting the *Tagetes* seedlings (time = 3 days) and its subsequent disappearance in the DGGE profiles raises several questions concerning the ecological functions and symbiotic relations between plants and microorganisms.

Finally, the sequence of nucleotides in Band B6 was similar to the group of bacteria of the genus *Delftia*, which has been characterized by its role and capacity to produce D-amino acids in the hemolymph of an insect called the glassy-winged sharpshooter (GWSS) (Bextine et al., 2010). Also, *Delftia* sp., together with other bacteria, such as *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum* and *Rhodococcus erythropolis*, have been identified as phosphate-solubilizing bacteria (PSB), thus confirming their ability to solubilize significant amounts of tricalcium phosphate through secretion of organic acids (Chen et al., 2006; Saharan and Nehra, 2011).

Although this band was only observed at 60 and 90 days and exclusively in the rhizosphere of *T. coronopifolia*, this does not preclude its possible existence in the other species of *Tagetes*. Its presence at these times could suggest an association with the process of floral initiation. Serrato-Cruz et al. (1998) determined that floral initiation of *T. patula* and *T. erecta* occurs 56 days after planting, and various studies have reported the use of experimental microorganisms and commercial isolates from rhizospheric soils that have the capacity to solubilize phosphates which positively promote the onset of flowering and an increase in the diameter and length of the flower in ornamental plants such as tuberoses (*nardo*) (Swaminathan et al., 1999), roses (Singh et al., 2003), carnations (Gupta et al., 2004), gladioli (Muzain et al., 2004), gaillardias (Deshmukh et al., 2008), and some varieties of African marigolds (Chandrikapure et al., 1999).

Conclusions

This investigation is a preliminary attempt to study the antagonistic effect of three species of the genus *Tagetes* in the bacterial communities of the rhizosphere. We found no changes in alpha diversity but a drastic change in Beta diversity trough time and among the three species of the genus *Tagetes*. The results presented herein support the selective effect of plant rhizospheres on microbial populations in the soil; an effect that can be highly-specific due that even within *Tagetes* each single species had a unique bacterial community. It is necessary in the future to perform experiments of rhizobacterial succession that imply other molecular techniques and quantification of the allelochemicals exuded by the roots and physicochemicals properties of the soil at different stages of plant development to

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evaluate if the community structure is driven by biotic or abiotic factors. The results found in this study could be an important finding due that it makes possible to determine and establish the optimum time for the application of biological controls or biofertilizers on commercial plants.

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EFFECTS OF GA_3 HORMONE TREATMENTS ON ION UPTAKE AND GROWTH OF PEPPER PLANTS UNDER CADMIUM STRESS

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Abstract. The present study was conducted to identify the response of pepper plants under cadmium stress to gibberellic acid (GA_3) treatments. Plants were exposed to different cadmium chlorine doses (0, 20, 40, 60 ppm) cadmium chloride ($CdCl_2$) and GA_3 (10 ppm) treatments. A resistance scale was used based on symptoms over the leaves and mineral element analyses (K, Cu, Zn, Fe, Mn, Mg and Cd) were performed. Experiments were conducted in hydroponic culture with Demre pepper cultivar under controlled conditions. Present results revealed significant effects of Cd treatments on Cd, Fe, Zn, Cu, Mn, Mg and K concentrations of the plants. Combined treatments of Cd and GA_3 (10 ppm) also increased ion accumulation especially in leaves. It was observed under stress conditions that GA_3 hormone treatments inhibited plant growth to prevent stress, increased ion uptake, well-adjusted ion balance and let the plants avoid from stressors.
Keywords: *cadmium, gibberellic acid, heavy metal stress, ion accumulation, pepper (*Capsicum annuum *L.)*

Introduction

Industrialization and urbanization bring about serious environmental pollution and exert significant threats on nature (Güven et al., 1999).

Even the trace amounts of heavy metals in air, soil and water resources may be dangerous for all living things. Agricultural and industrial activities may significantly increase especially cadmium (Cd) concentrations in the air surrounding us. The presence of heavy metals in trace amounts in soil, water and air can be dangerous to all living things, and the concentration of Cd in the environment is increasing due to agricultural and industrial factors (Foy et al., 1978). Such an increase is mostly resulted from anthropogenic sources and ultimately passed into the agricultural lands through sewage sludge treatments and fertilizer applications (Doğan and Saygıdeğer, 2009).

Cadmium may reduce germination, growth, development, yield and quality of various plants. Several studies were carried out to determine the threshold values and to investigate the morphological and physiological effects of Cd toxicity (Bertin and Averbeck, 2006).

Whether or not being essential element for plant growth, excessive heavy metal accumulation in plant tissues and organs negatively influence vegetative and generative organs of the plants (Gür et al., 2004). With toxic impacts, heavy metals may damage several physiological processes such as transpiration, stomatal conductance, enzyme activity, germination, protein synthesis, membrane stability and hormonal balance (Kennedy and Gonsalves, 1987). Toxicity may vary both from one metal to another and from one organism to another. Positive or negative (toxic) impacts not only depend on element type and concentration, but also closely related to genetic-based physiological responses of different species (Haktanır and Arcak, 1998).

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Cadmium stress reduces water and ion uptake of plants and hinders root growth and development. Stomata also close under cadmium stress, thus water loss through transpiration is reduced and cadmium transport is hindered (Salt et al., 1995). Cd accumulation may have toxic impacts on plants and damage mineral nutrition and carbohydrate metabolism of the plants, and then ultimately limits plants growth and development (John et al., 2009). Cadmium also inhibits chlorophyll biosynthesis and reduces total chlorophyll content (Stobart et al., 1985). Zinc and iron are essential micro nutrients for several biochemical processes in plants (Marschner, 1995). Cadmium interacts with these nutrients and directly influences nutrient uptake. Such interactions ultimately influence nutrient distribution, results in nutrient deficiency/imbalance and recesses plant growth and development (Zhang et al., 2002).

There is a consensus on stimulation of internal hormone levels through external application of different growth regulators (El-Shahaby et al., 1992). Rodriguez et al. (2006) indicated that the hormones in EP (extracellular products) produced by Cyanobacteria developed salt-tolerance in paddy seedlings. It was also indicated in the same study that GA treatments stimulated abscisic acid (ABA) production as a response to salt stress and altered and reduced the ratios of growth regulators.

The present study was conducted with pepper plants of Solanaceae family, mostly produced as early grown or summer plant in Turkey, to investigate: a) the effects of cadmium on metabolic activities of the plants; b) the response of plants and adaptation mechanisms developed against this pollutant; c) stress relief through gibberellic acid treatments; d) correlations of plant growth regulators with ion uptake and accumulation in plants.

Materials and Methods

Experiments for stress factors and plant growth were conducted in hydroponic culture in a climate cabin with a split air-conditioner to provide normal atmosphere.

Pepper seeds were sown in pumice-filled plastic germination containers (40x25x5 cm). Following the sowing of 100 seeds to each container, irrigation was performed with tap-water. Containers had 9 holes (0.5 cm) beneath to drain the irrigation water. Pumice was thoroughly wetted and excess irrigation water was drained. Then germination containers were placed in climate cabin with 25^{o}C temperature and 70% relative humidity. Containers were covered with moist paper and regularly controlled. Gradual tap water applications were continued to prevent the drying of pumice. Irrigations were started to be performed with Hoagland nutrient solution when the seedlings had horizontal cotyledon leaves and the first true leaves (Hoagland and Arnon, 1938). The seedlings with the 2nd true leaves were transplanted into hydroponic culture. Plastic cuvettes (25x25x18 cm) filled with Hoagland nutrient solution were used for hydroponic culture. Pepper seedlings were wrapped around with small sponge pieces and placed in specially designed platforms with holes over for each seedling. These platforms were placed over the cuvettes as to have the roots immersed in nutrient solution. Aeration was supplied through the nutrient solution with thin plastic hoses connected to two aquarium pumps.

Following the growth of seedling in hydroponic culture for two weeks, cadmium treatments were initiated. Experiments were conducted by completely randomized design with three replications with 20 plants in each replication. For cadmium treatments, 0, 20, 40, 60 ppm $CdCl_2$ doses were added to nutrient solution (1/2 Hoagland). Solutions were renewed weekly and care was taken to sustain the same

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cadmium concentrations in each renewal. Together with cadmium, 10 ppm gibberellic acid was applied to plants. Sampling for measurements and analyses were made 15 days after cadmium treatments. Samples were used to determine some plant growth parameters (green herbage fresh weight, number of leaves, plant heights, cadmium resistance scales based on leaf symptoms) and to determine some physico-chemical parameters (Cd, K, Cu, Zn, Fe, Mn, Mg contents).

A total of 8 different treatments were performed (control, cadmium 20, 40, 60 ppm, cadmium 20, 40, 60 ppm + gibberellic acid GA_3 (10 ppm).

Mineral element analysis

Three leaves from tip to downward were taken and they were kept in deep freezer at –40°C. About 200 g samples were taken from the deep freezer and samples were supplemented with 10 ml 0.1 N HNO_3 (Nitric acid). They were then kept in plastic boxes at dark and room temperature for a week. Samples were shaken in a shaker for 24 hours and resultant extract was subjected to K^+, Cu^+, Zn^+, Fe^+, Mn^+, Mg^+, Cd ion analyses in flame photometer (Eppendorf flame photometer). Fresh leaf ion concentrations were expressed in μg/mg fresh weight (Taleisnik et al., 1997).

Experiments were conducted in randomized plots design with 3 replications. Statistical analyses for plant growth parameters, ion and enzyme data were performed with SAS (1985) software.

Results

Data about plant growth parameters are provided in *Table 1*. While the greatest root weight was obtained from the control treatment, the lowest value was obtained from Cd3+GA_3 treatment. The same treatments had the similar characteristics for root lengths. Control treatment had the greatest stem weight and it was followed by GA_3 treatment. As compared to the control treatment, the greatest decrease was observed in Cd+GA_3 treatment, but it was placed in the same statistical group.

Table 1. *Plant growth and development parameters*

Applications	Root weight	Root height	Stem weight	Stem height	Leaf weight	Number of leaves	Total plant weight
	(g)	(cm)	(g)	(cm)	(g)	(number)	(g)
Control	2.303 A	19.833 A	1.778 A	11.833 A	6.670 A	9.500 A	10.751 A
Cd1+GA_3	1.220 C	10.550 CD	0.790 C	10.500 A	2.930 D	7.000 C	4.940 EF
Cd2+GA_3	1.991 AB	14.167 BC	0.713 C	9.667 A	2.571 DE	6.833 C	5.275 DE
Cd3+GA_3	1.075 C	9.333 D	0.615 C	10.167 A	1.900 E	6.000 C	3.590 F
Cd1	1.348 BC	15.833 AB	1.365 AB	11.000 A	4.483 C	7.333 BC	7.196 C
Cd2	1.510 BC	16.833 AB	1.323 B	11.000 A	3.733 C	7.333 BC	6.743 CD
Cd3	1.298 C	16.667 AB	1.311 B	10.167 A	2.898 D	7.066 C	6.908 CD
GA_3	1.686 A-C	14.167 BC	1.725 A	11.667 A	5.686 B	9.166 AB	9.097 B

Means indicated with the same letters in the same column are not significantly different.

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There were not any significant differences in stem lengths of the treatments. Control treatment had the greatest leaf weight. As compared to the control treatment, the greatest decrease was observed in Cd3+GA_3 treatment. The results for number of leaves were similar with the results for leaf weight. The greatest plant weight was observed in control treatment and the greatest decrease as compared to control treatment was observed in Cd3+GA_3 treatment. With regard to all growth parameters, it is remarkable that the lowest values were seen in Cd3+GA_3 treatment. Again as compared to the control treatment, leaf weight, number of leaves, total plant weight decreased with increasing cadmium doses.

As compared to control and single Cd treatments, Cd + GA_3 treatments increased root, stem and leaf Cd concentrations. The greatest leaf Cd accumulation in single Cd treatments was observed in leaves (*Table 2*). The greatest root cadmium concentrations were observed in Cd3+ GA_3, Cd2+ GA_3 treatments and they were followed by Cd1+ GA_3 treatment. The greatest stem cadmium concentration was observed in Cd3+ GA_3 treatment and it was followed by Cd2+ GA_3 and Cd1+ GA_3 treatments. The greatest root and stem cadmium concentrations were observed in Cd3+GA_3 treatments and increasing values were observed with increasing treatment doses. The same case was not valid for leaves; the greatest leaf cadmium concentration was obtained from Cd3 treatment and it was followed by Cd2 and Cd1 treatments.

Table 2. *Root, stem and leaf Cd concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Cd	Cd	Cd
Control	1.17 D	0.786 E	0.629 E
Cd1+GA_3	592.32 B	20.678 B	8.183 D
Cd2+GA_3	737.52 A	21.895 B	19.896 C
Cd3+GA_3	748.40 A	27.500 A	20.759 C
Cd1	438.16 C	5.373 D	27.895 B
Cd2	468.75 C	9.718 C	31.284 AB
Cd3	488.08 C	12.152 C	34.463 A
GA_3	1.14 D	0.505 E	0.447 E

Means indicated with the same letters in the same column are not significantly different.

There were remarkable differences in Fe contents of plant organs in different treatments (*Table 3*). While the greatest root Fe content was obtained from the control treatment, the greatest stem Fe content was obtained from GA_3 treatment and the greatest leaf Fe content was obtained from the control and GA_3 treatments. The lowest root and stem Fe contents were obtained from Cd3+GA_3 treatment and the lowest leaf Fe content was obtained from Cd1 treatment.

Considering the Zn contents of the roots, stems and leaves of treated plants, the greatest values were obtained from the control and GA_3 treatments and the lowest values were obtained from single Cd treatments. As compared to control and single GA_3 treatments, Zn contents decreased with Cd + GA_3 treatments, but increased with single Cd treatments (*Table 4*).

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Table 3. *Root, stem and leaf Fe concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Fe	Fe	Fe
Control	2007.17 A	67.868 C	117.069 A
Cd1+GA_3	1975.02 AB	70.118 C	83.241 B
Cd2+GA_3	1732.66 C	52.837 D	83.141 B
Cd3+GA_3	1234.88 E	37.097 E	51.767 D
Cd1	1456.66 D	31.742 E	35.582 E
Cd2	1535.60 D	31.872 E	47.357 D
Cd3	1728.47 C	37.438 E	60.662 C
GA_3	1815.32 BC	92.548 A	123.345 A

Means indicated with the same letters in the same column are not significantly different.

Table 4. *Root, stem and leaf Zn concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Zn	Zn	Zn
Control	55.601 A	9.5197 A	9.6410 BC
Cd1+GA_3	50.569 AB	8.5043 B	8.3917 BC
Cd2+GA_3	50.833 AB	8.9163 AB	8.7853 BC
Cd3+GA_3	48.915 AB	7.0087 C	8.3247 BC
Cd1	33.755 DE	4.6890 D	5.9730 D
Cd2	39.789 CD	4.2420 D	5.9220 D
Cd3	35.123 DE	4.9230 D	5.7107 D
GA_3	56.944 A	9.7750 A	9.2063 AB

Means indicated with the same letters in the same column are not significantly different.

With regard to Cu accumulation in roots, stems and leaves of pepper plants, similar with Fe and Zn, the greatest values were observed in control and GA_3 treatments without Cd treatments. As compared to control treatment, decrease was observed with Cd + GA_3 treatments; however Cu accumulation levels were lower in single Cd treatments (*Table 5*).

Considering Mn accumulation in roots, stems and leaves of pepper plants, the greatest values were observed in control and GA_3 treatments. As compared to control treatment, Cd + GA_3 treatments generally decreased Mn contents of roots and leaves, but yielded higher Mn contents than single Cd treatments. Cd+GA_3 treatments yielded the same stem Mn contents with the control treatment, but higher than Cd treatments (*Table 6*).

Table 5. *Root, stem and leaf Cu concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Cu	Cu	Cu
Control	13.379 A	3.6810 A	3.8047 A
Cd1+GA_3	10.021 BC	1.8233 CD	3.0947 AB
Cd2+GA_3	11.060 B	2.3680 BC	2.6833 B
Cd3+GA_3	9.781 BC	2.8907 B	2.7610 B
Cd1	8.319 C	1.4003 DE	2.7590 B
Cd2	8.962 BC	1.1940 E	2.3107 C
Cd3	8.660 BC	1.6737 DE	2.1593 C
GA_3	12.549 A	3.7523 A	3.7723 A

Means indicated with the same letters in the same column are not significantly different.

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Table 6. *Root, stem and leaf Mn concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Mn	Mn	Mn
Control	265.12 A	10.4447 A	25.054 A
Cd1+GA_3	180.62 BC	10.7663 A	22.349 B
Cd2+GA_3	256.66 A	10.3053 A	23.168 B
Cd3+GA_3	202.31 B	10.3833 A	22.392 B
Cd1	98.69 D	5.8190 B	11.960 D
Cd2	109.17 D	6.8473 B	11.721 D
Cd3	98.34 D	6.8153 B	15.042 C
GA_3	243.89 A	10.8620 A	27.505 A

Means indicated with the same letters in the same column are not significantly different.

Control treatments had the greatest root Mg contents. However, contrary to other ion concentrations, single GA_3 treatments had the least Mg accumulation levels. Cd + GA_3 treatments also had lower Cd accumulations than single Cd treatments. With regard to stem Mg contents, Cd+GA_3 treatments were placed in the same statistical group with control treatments, but single Cd treatments had lower stem Cd contents and were places in different statistical group. As it was in other ions, the differences in root, stem and leaf Mg contents of different cadmium doses were not found to be significant (*Table 7*).

Table 7. *Root, stem and leaf Mg concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	Mg	Mg	Mg
Control	61.354 A	30.467 A	27.889 A
Cd1+GA_3	24.376 CD	29.546 AB	29.322 A
Cd2+GA_3	22.798 D	30.160 AB	27.485 A
Cd3+GA_3	23.237 D	26.924 B	23.255 B
Cd1	38.136 B	13.390 D	21.836 B
Cd2	31.969 BC	17.091 C	22.644 B
Cd3	29.839 BC	16.864 C	23.536 B
GA_3	19.182 D	29.389 AB	28.706 A

Means indicated with the same letters in the same column are not significantly different.

Table 8. *Root, stem and leaf K concentrations (µ g/mg T.A.)*

APPLICATIONS	ROOT	STEM	LEAF
	K^+	K^+	K^+
Control	306.82 A	339.97 A	317.73 A
Cd1+GA_3	157.68 B	327.60 A	284.20 AB
Cd2+GA_3	164.41 B	334.99 A	278.43 AB
Cd3+GA_3	158.21 B	325.24 A	278.09 AB
Cd1	126.95 CD	230.64 B	259.45 BC
Cd2	134.78 C-D	224.87 B	214.04 C
Cd3	132.69 C-D	239.86 B	216.80 C
GA_3	160.34 B	331.72 A	331.36 A

Means indicated with the same letters in the same column are not significantly different.

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Potassium (K) contents of root, stem and leaf samples are provided in *Table 8*. The greatest root K content was obtained from the control treatment and it was respectively followed by Cd+GA_3 treatments and single GA_3 treatment. The least root K content was obtained from single Cd treatments, but the differences between Cd doses were not significant. The differences in stem and leaf K contents of different Cd doses were not also found to be significant. Only the differences between Cd and GA_3 treatments were found to be significant.

Discussion

Current findings revealed that cadmium significantly hindered root, stem and leaf growth of pepper seedlings. However, differences in plant growth parameters of different cadmium doses were not found to be significant. Despite the insignificant differences in stem lengths, Cd + GA_3 treatments inhibited plant growth and development. Current findings about the effects of cadmium comply with results of various earlier studies carried out with different plants. It was previously reported that cadmium inhibited root and stem growth of *Vigna unguiculata* L. var. Pusa falguni plants (Nagor, 1997); cadmium, copper, lead and zinc reduced root and shoot development of *Sorghum bicolour* L. plants (Pandit and Prasannakumar, 1999). Cadmium and nickel reduced root and stem lengths of *Oryza sativa* L. cv. Bahia plants (Moya et al., 1993); zinc reduced stem lengths of Brassica juncea seedlings (Prasad et al., 1999); cadmium also reduced stem and root lengths of *Zea mays* L. Dekalp cv. 73 Sponsor plants (Rascio et al., 1993). Similarly, Zengin and Munzuroğlu (2003) investigated the effects of cadmium ($CdCl_2.H_2O$) on root, stem and leaf growth of bean seedlings and reported that cadmium significantly hindered root, stem and leaf growth and inhibitions were parallel to increasing cadmium doses. As compared to control treatment, plant growth was slow in Cd+GA_3 treatments. Yasar et al. (2016) applied GA_3 to eggplant seedlings under salt stress and reported selective ion uptake of plants. As reported by Ashraf et al (2001), GA_3 might have reduced nitrogen uptake of the plants. Combined application of GA_3 and cytokine-like growth regulators may provide positive contribution in elimination of salt stress (Xiong et al., 2002). Lin and Kao (1995) and Rodriguez et al. (2006) also reported that GA_3 treatments reduced the growth inhibition of paddy and some other plants.

Cd treatments increased root, stem and leaf Cd concentrations as compared to control and single GA_3 treatments. Cd accumulation in roots and stems were higher in Cd + GA_3 treatments than in single Cd treatments. However, an inverse case was valid for leaves. It was observed that GA_3 prevented toxic ion transport to leaves to prevent heavy metal toxicity. Several researchers reported increasing plant Cd concentrations with increasing Cd doses. Bachir et al. (2004) reported increasing plant Cd concentrations with Cd treatments in cotton; Safarzadeh et al. (2013) reported the same case in paddy plants. Tiryakioğlu et al. (2006) reported that increasing Cd doses increased green herbage and especially root Cd concentrations, majority of cadmium taken up by the plants accumulated in roots and slight amounts were transported to green herbage.

Cadmium treatments significantly reduced Fe, Zn and Cu concentrations of all plant organs. However, as compared to single Cd treatments, Cd+GA_3 treatments increased Fe, Zn and Cu accumulations in all three plant organs. Single GA_3 treatments also increased stem and especially leaf Fe, Zn and Cu accumulations as

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compared to single Cd treatments. Similarly, Bachir et al. (2004) reported that 0.1 and 1 µM Cd treatments increased Fe, Zn and Cu concentrations of cotton plants and indicated a potential synergic effect of Cd on Fe and Zn uptake. Köleli et al. (2004) reported under Zn-deficient conditions that increasing Cd treatments reduced green herbage Fe concentrations; however, Cd treatments did not have significant effects on green herbage Fe concentrations under Zn-sufficient conditions. Safarzadeh et al. (2013) also reported that Cd treatments significantly reduced green herbage Zn and Fe concentrations. Wu et al. (2004) carried out a study about the effects of cadmium on micro element uptake and transport in cotton plants and reported that 0, 0.1 and 1 µM Cd treatments did not change leaf Fe, Zn and Cu concentrations significantly, but 10 µM Cd treatment significantly increased leaf micro element concentrations. Amal et al. (2014) applied GA_3 to barley plants under salt stress and reported decreasing Zn, Fe, Co, Pb, Cr, Cd and Mn ion accumulation under salt stress and indicated that GA_3 treatments relieved the impacts of salt stress and increased ion uptake levels. Akman (2009) also reported similar results for Fe, Zn and Cu ion accumulation in wheat.

As it was in other ion accumulations, Cd and GA_3 treatments yielded similar results for K, Mn and Mg accumulations. Cd treatments reduced the accumulation levels of all ions, except for Cd; GA_3 treatments also yielded slight decreases in accumulation of all ions again except for Cd as compared to control treatment, but increased ion accumulation as compared to single Cd treatments. Mohamed and Gomaa (2012) and Amal et al. (2014) carried out a study with barley plants under salt stress and reported that GA_3 treatments reduced K, Mn and Mg ion accumulation levels in plants and GA_3 treatments also reduced the impacts of salt stress and increased ion uptakes. Similarly, Schachtman and Lio (1999) in barley and Iqbal and Ashraf (2013) in wheat plants under salt stress, reported that salt stress reduced ion uptake in GA_3 treatments, but GA_3 treatments improved ion uptake of plants under salt stress.

It was concluded based on current findings that combined GA_3 and Cd treatments did not have positive contributions to plant growth, but plant under these treatments behaved selectively in ion uptakes to prevent from toxic impacts of Cd. Such impacts of growth regulation hormone GA were also reported by previous researchers. When applied to plants under abiotic stressors, GA inhibited plant growth through synthesis of DELLA proteins. GA treatments increase the activity of this protein and may have positive contributions to stress tolerance of the plants since it inhibits plant growth under abiotic stress conditions (Achard et al., 2006; Achard et al., 2008a; Magome et al., 2008). Increased DELLA activity with GA treatments limits the accumulation of reactive oxygen species (ROS) under Cd stress, thus prevents cell deaths (Achard et al., 2008b). DELLA proteins were reported as behaved like inner-cell suppressors of GA-induced metabolic activities (Peng et al., 1997; Silverstone et al., 1998; Ogawa et al., 2000; Ikeda et al., 2001; Chandler et al., 2002).

Conclusion

When we evaluate the data we obtained without working, we found that the GA_3 application increased ion uptake in plants, GA_3 treatment with Cd inhibited plant growth, but Cd and GA_3 treatments did not cause damage to plants caused by strhenogenesis, even though Cd stress was applied to plants. The reason for this is that it may be originated from DELLA proteins that inhibit enzyme activity when GA_3 is

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applied to plants at the time of stress. Enzyme activities and DELLA proteins should be looked for in order to be fully illuminated. In addition, after application of GA_3 at the time of stress, the levels of organic acid in the cell should be monitored.

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THE EFFECT OF CHEMICAL AND NON-CHEMICAL CONTROL METHODS ON WEEDS IN POTATO (*SOLANUM TUBEROSUM* L.) CULTIVATION IN ARDABIL PROVINCE, IRAN

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Abstract. Potato is one of the most important crops in Iran and on average, this crop is affected annually by weeds damage which must be prevented by various control methods. Crop residues and other types of mulches are non-chemical methods of weed control in sustainable agricultural systems. The aim of present study was to investigate the effect of chemical and non-chemical control methods on weeds in potato crop. An experiment was conducted in 2015 in two agricultural research stations of Alarogh and Samian in Ardabil city in a randomized complete block design with three replications. The treatments were as follows: 1) Trifluralin herbicide, 2) Metribuzin herbicide, 3) cultivator, 4) wheat straw mulch, 5) canola straw mulch, 6) black plastic mulch, 7) transparent plastic mulch 8) weed infested and 9) weed free. Based on results, all treatments were affected by weed control methods ($P \leq 0.01$), so that Shannon-Wiener index treatment was ($P \leq 0.05$). The greatest diversity of Shannon-Wiener index measured in present experiment was observed in weed infested treatment (average comparison by Duncan test). The treatments of Trifluralin herbicide, cultivator and transparent plastic mulch after the weed infested treatment had the highest species diversity. The lowest density of weeds was related to the wheat straw mulch. Most weeds density was counted as 99.1 plants/m^2 of weed infested treatment. The wheat straw mulch deceased weeds density as 84% compared to weed infested. The canola straw mulch also was deceased weeds density as 79% comparing to weed infested. However, weed biomass in the treatments of transparent and black plastic mulch compared to the weed infested treatment had the highest weeds biomass as 48 and 40%, respectively. The lowest weed biomass was observed in canola and wheat mulch by 16 and 18% after the weed infested. It can be conducted that, the use of plant mulches or residues can provide more proper control of weeds compared to the use of herbicide and the use of plant mulches reduces use of herbicides.
Keywords: *biomass, canola, density, diversity, herbicide, plant mulch, plastic mulch, wheat*

Introduction

In most potato producer countries in the world such as China, India, Russia, Ukraine, America, Germany, Bangladesh, etc. with different climatic conditions, weed control is carried out by agricultural, mechanical and chemical methods or their combination in potato fields. (Hutchinson et al., 2011; Kunz et al., 2015; Weber et al., 2016). Metribuzin (Sencor) is an herbicide belonging to Triazine family that is used in potato fields both

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before planting and pre-emergence potato, mostly to control annual grass and broad leaf weeds (Zand et al., 2007; Zaki et al., 2014). The use of Trifluralin (Treflan) is effective as pre- emergence in hilling step to control weeds of potato crop (Sheikhi et al., 2012; Borzouei et al., 2013). The use of tillage or cultivator along with herbicide, control of weeds effectively and it is used as one of the major methods of weed control in potato crop (Mohammaddoust et al., 2011). Nowadays, an organic method that is used in weed management is the application of mulches. Moreover, the application of herbal residues or plant mulch has a great importance to develop and expand sustainable farming systems. The need to increase farm productivity and profitability is a factor that stimulates the development of methods based on the ecological management of weeds. Considering that in industrialized countries as well as developing countries, increasing the inputs cost has been more than the products cost, so that has been endangered the economic support for farmers. If the better ecological process is used in weed management, agricultural income will be increased by reducing the inputs cost and this topic helping farmers to provide market friendly agricultural products (Dejam et al., 2010; Jafari et al., 2013; Najafi et al., 2016). Specifically, when using a weed control method, the competition of weeds with crop plants is reduced and as a result the use of other methods (as chemical control) to weed control is reduced. The purpose of this experiment was to determinate the best method that prevents weed growth and competition of weeds with crop.

Materials and Methods

Study area

The present experiment was conducted in 2015 at two stations: 1. Alarogh Agricultural Research Stationin (*Fig. 1*) Ardabil Province, Iran with an altitude of 1350 meters above sea level, a longitude of 48°, 20', a latitude of 38°, 15', a semi-arid and cold climate, and a soil pH of 7.6, and 2. Samian Agricultural Research Station (*Fig. 1*) in Ardabil Province, Iran with an altitude of 1320 meters above sea level, a longitude of 48°, 15', a latitude of 38°, 23', a semi-arid and cold climate, and a soil pH of 7.5-8. Two areas of Alarogh and Samian in Ardabil province are major areas under potato cultivation and many researches are being made on potato crop in these areas. Laboratory measurements were performed at the faculty of agricultural sciences and natural resources, university of Mohaghegh Ardabili, Ardabil, Iran.

Figure 1. *The images of the farm stations in Alarogh (Left) and Samian (Right)*

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Experimental design and treatments

The present study employed a randomized complete block design with three replications. The treatments were as follows: 1) Spraying Trifluralin herbicide on the soil between the rows of potato plants (75% EC was formulated with the amount of 2 L/ha by the Matabi model with an 8001 nozzle and the constant speed and pressure of sprayer in all treatments based on the 250 L/ha spraying; thus, the Trifluralin was mixed with soil at the depth of 0-10 cm) immediately after the second hilling of potato plants 45 days after planting the potatoes. 2) Spraying Metribuzin herbicide on the soil between the rows of potato plants with the amount of 1000 g/ha (with the formulation of 70% WP and sprayer specification such as the sprayer used for Trifluralin herbicides) immediately after the second soil hilling of potato plants 45 days after planting the potatoes. 3) Cultivator practice once for 20 days after the second hilling of potato plants 65 days after planting the potatoes. 4) Wheat straw mulch with the amount of 5 ton/ha and thickness of 15 cm immediately after the second soil hilling of potato plants 45 days after planting the potatoes. 5) Canola straw mulch applied similar to WH treatment. 6) The application of black plastic mulch, covering the space between the rows with plastic sheets with the thickness of 50 microns immediately after the second soil hilling of potato plants 45 days after planting the potatoes in the plot or row. 7) The application of transparent plastic mulch was similar to that of BPL treatment. 8) Weed infested or no weed removal in the entire growing season. 9) Weed free or weed removal in the entire growing season in both stations.

Land preparation and planting

The land was prepared for planting by secondary plowing immediately after favorable weather and soil conditions. On 5th June 2015, potato tubers (a variety of Agria) were hand-planted in rows, between which there was a distance of 75 cm and a distance of 25 cm on the ridge (the distance between the tubers planted on the planting row in each plot). Each plot had an area of 3 × 3.5 m.

After selecting the locations of experiment and before the preparation actions, 10 points of each farm soil were randomly sampled to provide composite samples and to analyze soil. Then, based on the results of soil analysis, fertilization was performed with application of phosphate fertilizers (Triple superphosphate with amount of 178 kg.ha^{-1} at two steps based on recommendation of research stations, 50 percent at planting time and 50 percent during the formation of tubers) and nitrogen (urea with amount of 300 kg.ha^{-1} at three times, 25 percent during potato planting, 50 percent during emergence and 25 percent immediately after formation of tubers). Irrigation was also conducted after the first step of soil irrigation and emergence of plants with a 7-day interval. Two potato hilling steps were done for at the base of plants using a hoe at 25 and 45 days after potato planting, respectively.

Potato and weed sampling

At the end of the growing season and a month before harvesting potato tubers (September), the weeds were sampled according to species and were counted. Weed sampling were performed in each plot with dimensions of 0.5 × 0.75 square cm of the soil surface and were transported to the laboratory. After counting the number of plants by

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species, shoots of weed of each species were separately and put in special bags and placed at oven for 72 hours at 75 ◦C and after drying completely, its were weighed with a balance with precision of 0.001g. Before performing statistical analysis and for data uniformity, conversion of $\sqrt{X+0.5}$ was used for data related to density and dried weight of weed. In order to determine the potato yield, after completion of the growing season and ripening potato tubers, the middle two rows of plants (inside plots) were collected during a meter entirely.

Shannon-Wiener index

In order to estimate weed species diversity, Shannon-Wiener index were used. Shannon-Wiener index was used to calculate the following equation:

$$H' = -\Sigma(n_i/N)(Log_2 n_i/N) \quad \text{(Eq. 1)}$$

In the mentioned equation, H' is Shannon-Wiener index, $(0 \leq H')$, n_i is the number of i-th species and N is the number of peoples (Shannon and Wiener, 1949). The data analysis was finally performed using SAS ver. 9.1 software and graphs were drawn by Excel ver. 2013 software.

Results and Discussion

Weed species diversity and density

In this study, among the 12 weeds species observed in potato field were the seven species annual weeds and five species perennial weeds. The weed species were include Common Amaranth, lamb's quarters, Bindweed, Green foxtail, Prickly lettuce, Russian knapweed and Milk thistle had the highest weed prevalence of dominant (*Table 1*).

Table 1. *The biological and Photosynthetic traits of weed species observed in the sampling units of potato farm*

row	Species name	Scientific name	Family	Life cycle	Photosynthetic cycle
1	Comon Amaranth	*Amaranthus retroflexus*	Amaranthaceae	Annual	C4
2	Russian knapweed	*Acroptilon repens*	Asteraceae	Perennial	C3
3	creeping thistle	*Cirsium arvense*	Asteraceae	Perennial	C3
4	Prickly lettuce	*Lactuca seriola*	Asteraceae	Annual	C3
5	Wild mustard	*Sinapis arvensis*	Brassicaceae	Annual	C3
6	Common Lamb's Quarters	*Chenopodium album*	Chenopodiaceae	Annual	C3
7	Bindweed	*Convolvulus arvensis*	Convolvulaceae	Perennial	C3
8	Pigweed	*Echinochloa crus galli*	Poaceae	Annual	C4

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9	Green foxtail	*Setaria viridis*	Poaceae	Annual	C3
10	Licorice	*Glycyrrhiza glabra*	Papilionaceae	Perennial	C3
11	Tumbleweed	*Salsola kali*	Chenopodiaceae	Annual	CAM
12	Milk thistle	*Sonchus arvensis*	Asteraceae	Perennial	C3

In present study the average of Shannon-Wiener diversity index in both stations was changed ($P \leq 0.05$) by changing of management practices for weed management (*Table 2 and Fig. 2*). The greatest diversity of Shannon-Wiener index measured in present experiment was observed in weed infested treatment (*Fig. 2*). Blackwell (2011) stated that failure to weeds control in arable land or fallow cause of increase the density of weeds and also can be effective on weeds species diversity. According to the Shannon-Wiener index the treatments of Trifluralin, cultivators and transparent plastic mulch after the weed infested treatment had the highest species diversity (*Fig. 2*). The light passes through transparent plastic mulch and is likely to stimulate the germination of weeds but because of the low temperature in present region (Experiment locations) could not prevent weeds germination and the result, may be stimulated weed species seeds and causes of weed germination in the amplitude and so may be effective on the composition and diversity of weeds. Majd et al. (2014) also achieved to similar results in this regard.

Table 2. *Combined statistical analysis (ANOVA) of the effects of experimental treatments on weeds biomass, density and Shannon -Wiener diversity index in potato cultivation*

		Mean of squares (MS)						
		Weed biomass			Weed density			
(S.O.V)	Df	Total	Grass	Broad leaf	Total	Grass	Broad leaf	Shannon-Wiener index
Station	1	3.3^{ns}	3.9^{ns}	9.6^{ns}	1.5^{ns}	4.5^{ns}	9.6^{ns}	0.4^{ns}
Rep (Station)	4	22.2	1.1	24.8	2.3	0.5	24.8	0.03
Treatment	7	56.1**	7.9**	56.9**	21.3**	8.0**	56.9**	0.3*
Station × Treat	7	5.1^{ns}	1.3^{ns}	5.4^{ns}	0.8^{ns}	1.1^{ns}	5.4^{ns}	0.1^{ns}
Error	28	8.5	1.8	9.8	1.4	1.5	9.8	0.1
CV	-	30.6	52.1	34.6	20.1	49.1	34.6	31.7

ns, non-significant difference. **, difference in level 1 percent. *, difference in level 0.5 percent.

In the farms, the composition, diversity and species richness simultaneously is affected by many factors including climate, soil properties, agricultural products, agricultural operations, tillage, cultivator and farming characteristics (Lososova et al., 2008; Azad et al.,

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2015). The lowest weed species diversity based on Shannon-Wiener index similarly were related to treatments of canola straw mulch, Metribuzin herbicide and black plastic mulch (*Fig. 2*). One reason for the decline of species diversity as a result of the use of mulch, is reducing amount of light reached into weed canopy, because increasing the coverage layer of mulch, more percent of light absorbed by crops or by mulch and therefore the remaining amount of light is reduced to weeds consumption as a result of reduced weed density and diversity (Dvorak et al., 2012; Azad et al., 2015).

Table 3. *Combined statistical analysis (ANOVA) of the effects of experimental treatments on tuber yield of potato*

		Mean of squares (MS)
(S.O.V)	**Df**	**Potato tuber yield**
Station	1	4.5^{ns}
Rep (Station)	4	0.5
Treatments	8	
Station × Treat	8	8.0**
Error	32	1.1^{ns}
CV		

ns, non significant difference **, difference in level 1 percent.

Brassica plant debris in addition to preventing light from reaching weed seeds and prevent the germination of weed spectrum like some monocots may reduce the species diversity also Allelochemicals released by the organs of these plants (*Brassicas*) may be effective on weed species composition (Pawlonka et al. 2015). The use of herbicides as a result of a specific range of weed control can be reduced weed diversity (Channappaguar et al., 2007; Nikolic et al., 2013). Based on results obtained from the combined statistical analysis of traits in present study, the treatments had a significant effect (P≤ 0.01) on weeds density in both stations (*Table 2*) so that the lowest total weeds density was obtained in wheat straw mulch and then, Metribuzin herbicide. The maximum total weeds density was counted as 99.1 plants per square meter in the weed infested treatment and the lowest total weeds density were related to the wheat straw mulch as 15.5 plants per square meter (*Fig. 3*).

The results showed that the use of wheat straw mulch decreased total weed density by about 84% comparing to weed infested treatment (*Fig. 3*). Canola straw mulch also decreased total weed density comparing to weed infested treatment by about 79% so that weed density difference in canola straw mulch compared to weed infested treatment was significant at (P≤ 0.01) (*Table 2*), however in terms of reducing the number of weeds it was less successful than wheat straw mulch, although this difference between two mentioned treatment was not significant (*Fig. 3*). Weed density reduction in plots where herbal mulch were used, may be because of inhibition of weed germination and growth at this stage of potato growth or as result of the release of Allelopathic substance, the results of the research of many researchers (Dhima et al., 2006; Judice et al., 2007) also showed that plant mulches prevent weed germination or growth with preventing the penetration of light or the release of Allelopathic substances.

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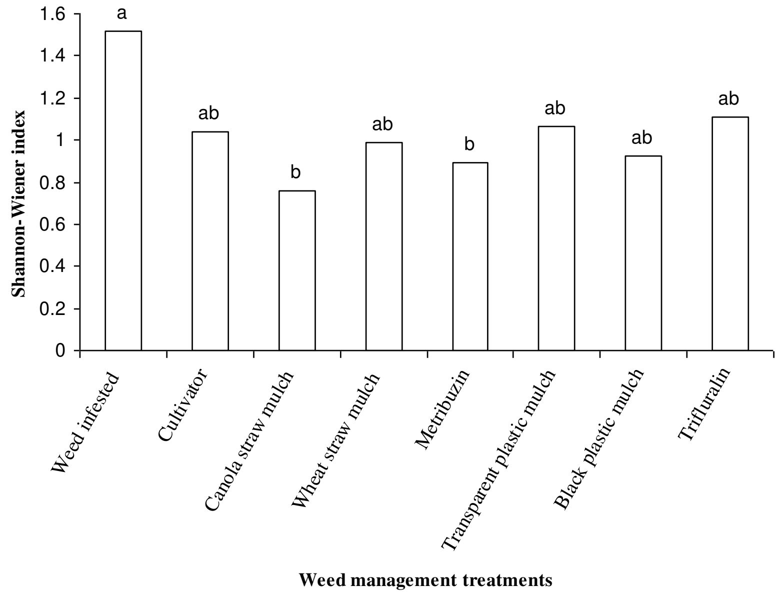

Figure 2. *The effect of different weed management treatments application on Shannon-Wiener diversity index in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

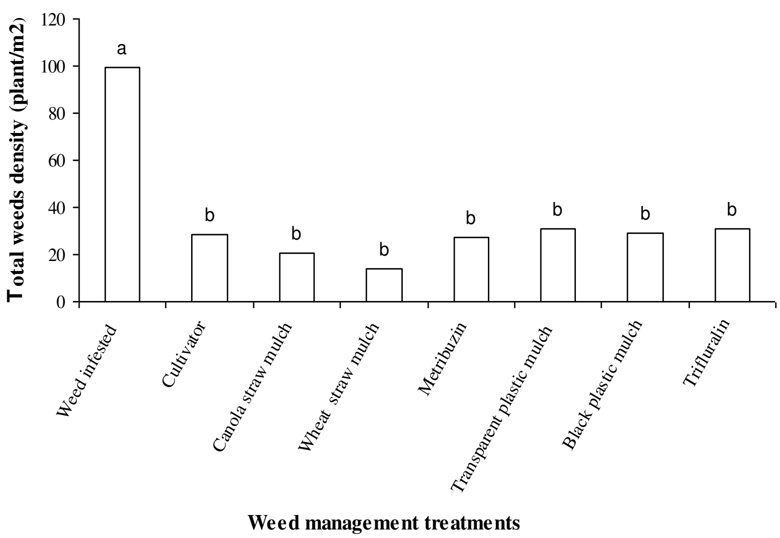

Figure 3. *The effect of different weed management treatments on total weed density average (plant/m^2) in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

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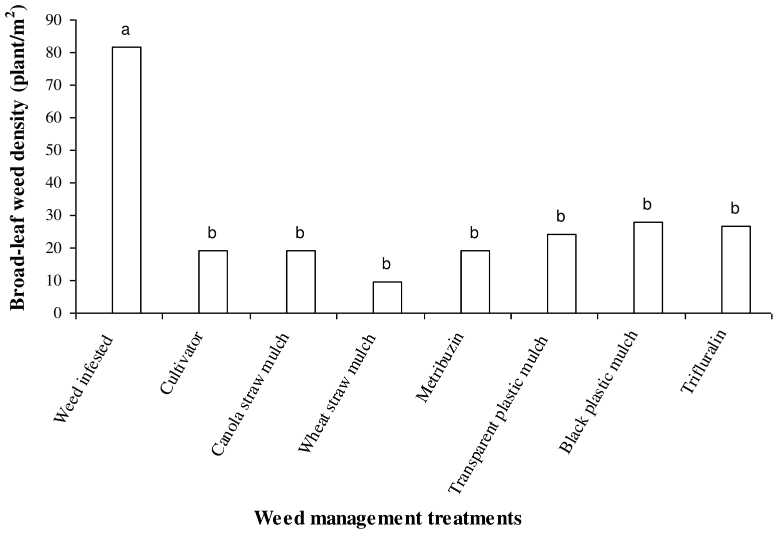

Figure 4. *The effect of different weed management treatments on Broad-leaf weeds density average (plant/m^2) in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

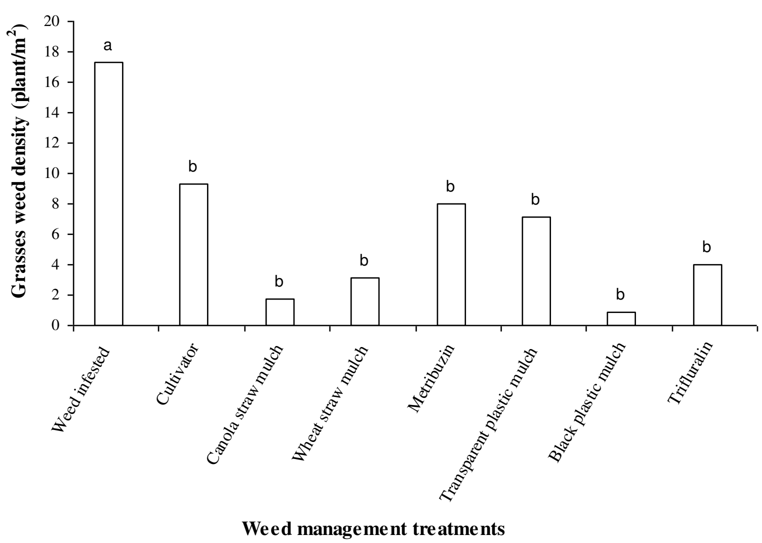

Figure 5. *The effect of different weed management treatments on Grass weeds density average (plant/m^2) in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

Moreover, Dhima et al. (2006) reported when applying the straw mulch of barley, Triticale and Rye in sugar beet crop, the germination of Pigweed (*Echinochloa crus galli*), were 69% less than the plots without plant residues. Herbicides had also a significant impact at (P≤ 0.01) on weeds density of potato cultivation (*Table 2*). *Figure 3* shows that total weed density was lower in Metribuzin and Trifluralin treatments compared to the

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weed infested treatment. At Metribuzin and Trifluralin treatments, total weed density was less than the weed infested treatment as 72 and 69%, respectively. Hence these differences were significant at (P≤ 0.01) (*Table 2*). Although the Metribuzin herbicide after wheat straw mulch had the greatest impact in reducing weed density of potato crop and showed a better performance than the Trifluralin herbicides to prevent from germination and growth of weed during the cultivation period, it had no significant differences with mentioned treatments in terms of reducing weed density. Channappaguar et al. (2007) showed among the herbicides of Alachlor, Pendimethalin, Diuron and Metribuzin, the Metribuzin had maximum efficiency in controlling broadleaf and grass weeds of potato crop because this herbicide effectively prevents of weeds germination and so control weeds. Black and transparent plastic mulch significantly controlled total weed density, the black color of plastic prevents reaching full light to light-dependent weeds for their germination and this point indicates the inhibitory effect of black plastic on the germination and growth of weeds (Majd et al., 2014). Density of broadleaf weeds species, as most important weeds in potato cultivator in all mulch treatments, cultivator and herbicides was reduced compared to weed infested treatment (*Fig. 4*). The maximum density of Comon Amaranth (broadleaf weed specie) has been observed in treatments of black plastic mulch, Trifluralin and transparent plastic mulch, hence the lowest density was observed in treatments of wheat straw mulch and Metribuzin herbicides application (*Fig. 4*). The black plastic mulch in some cases during the growing season is decomposed by sunlight and it may not completely effective to prevent of weed germination as a result of irrigation, wind and other factors, and gives the growth and germination opportunities to weed seeds (Haidar and Sidahmed, 2000; Majd et al., 2014; Zhang et al., 2015). Lamb's quarters weed (broadleaf weed specie) had the highest density in the application of herbicides of Metribuzin and transparent plastic mulch and the minimum density in treatments of cultivator and wheat straw mulch (*Fig. 4*). The use of Poaceae residues as mulch is one of the ecological agriculture components that well prevents of weed seeds germination (Maldonado et al. 2001; Shiyam et al., 2011; Didon et al., 2014). In present experiment the highest grass weed density was related to the treatment of cultivator, however the least one was related to the treatments of black plastic mulch, canola straw mulch and wheat straw mulch (*Fig. 5*). Various experiments have shown that different treatments such as herbicides and plant residues affecting on weed species composition in different ways (Dhima et al., 2006). However in the present study, density of broadleaf weeds was more than grass weeds, in other words, the highest density of weeds was related to the broadleaf weeds. Moreover, all treatments could significantly reduce the level of broadleaf and grass weeds compared to weed infested treatment.

Weed biomass

The results of experiment showed that the use of herbicides had a significant effect on the weeds biomass at (P≤ 0.01) (*Table 2*).

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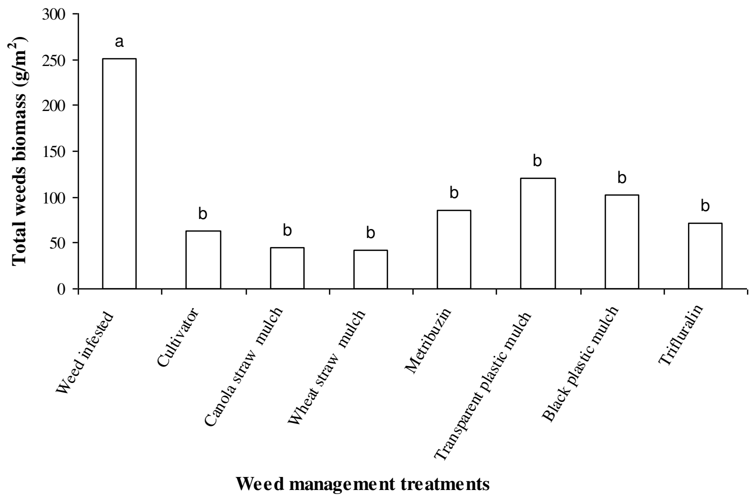

Figure 6. *The effect of different weed management treatments on total weed biomass average (g/m^2) in potato cultivation (The averages of at least one similar letter are not significantly different at* ($P \leq 0.05$)*, using Duncan's multiple range test)*

In present experiment, total weed biomass in the treatments of transparent and black plastics was 120.3 and 101.7 g/m^2, respectively, that showed the maximum biomass value comparing to the weed infested treatment (With biomass of 250.92 g/m^2) comparing to other treatments (*Fig. 6*), although the use of plastic mulches showed no significant difference with the use of herbicides and plant mulchs. However, the least amount of weed biomass was observed in treatments of wheat and canola mulches and it was found that these treatments can reduce weed biomass compared to weed infested treatment by a rate of 83% and 81% decrease, and this reflects the ability of plant residues in suppressing weeds and preventing their growth as well as preventing the spread of weeds and reducing the crop yield (Duppong et al., 2004). Plant residues are not only affect the soil but also can affect the germination, survival, growth and competitive ability of weed and crop plants (Majd et al., 2014; Pawlonka et al., 2015; Azadbakht et al., 2017). Although in current study, the lowest biomass of broadleaf and grass weeds showed a significant difference with the weed infested treatment and broadleaf weeds had more biomass than grass weeds (*Fig. 7* and *8*). Although, all chemical and ecological management treatments could statistically reduce broadleaf weed biomass compared to the weed infested treatment significantly, considering no significant difference between treatments of mulch and herbicides in terms of weeds biomass, it can be noted that the use of plant mulches could be more costly than the use of herbicides due to labor costs and costs related to mulch preparation.

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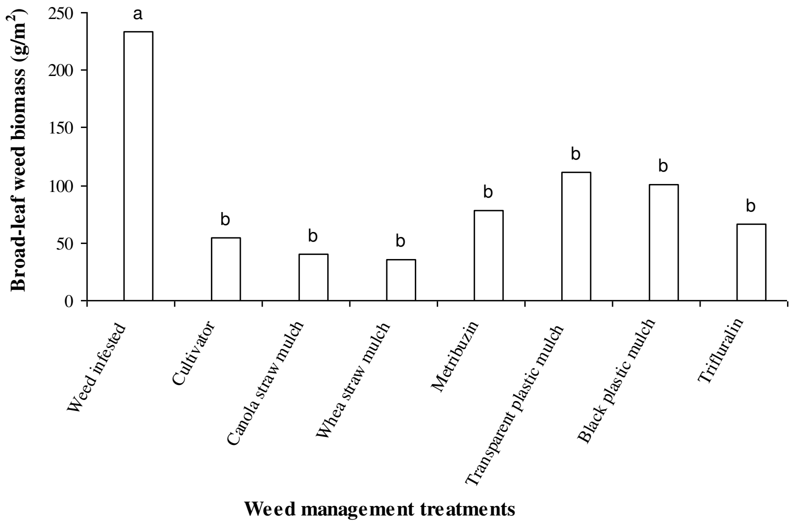

Figure 7. *The effect of different weed management treatments on Broad-leaf weeds biomass average (g/m²) in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

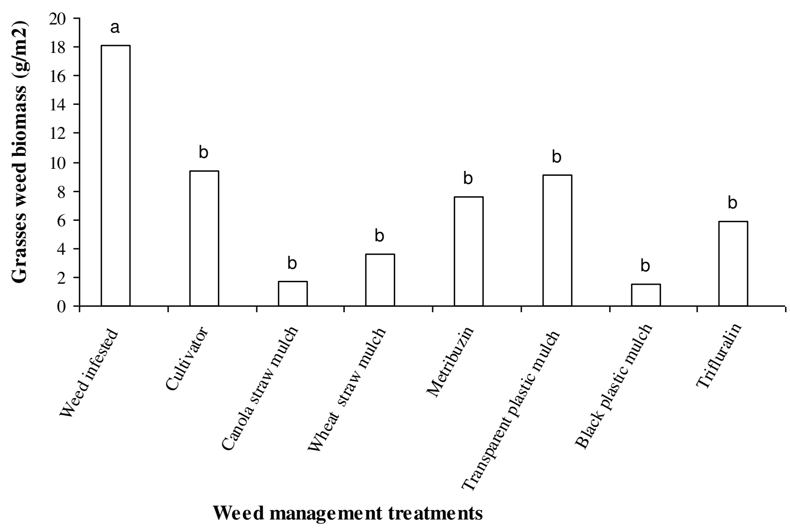

Figure 8. *The effect of different weed management treatments on Grass weeds biomass average (g/m²) in potato cultivation (The averages of at least one similar letter are not significantly different at* (P≤ 0.05)*, using Duncan's multiple range test)*

Potato tuber yield

The potato yield was significantly affected (P≤ 0.01) by the applied treatments in the experiment (*Table 3* and *Fig. 9*) except transparent plastic mulch treatment.

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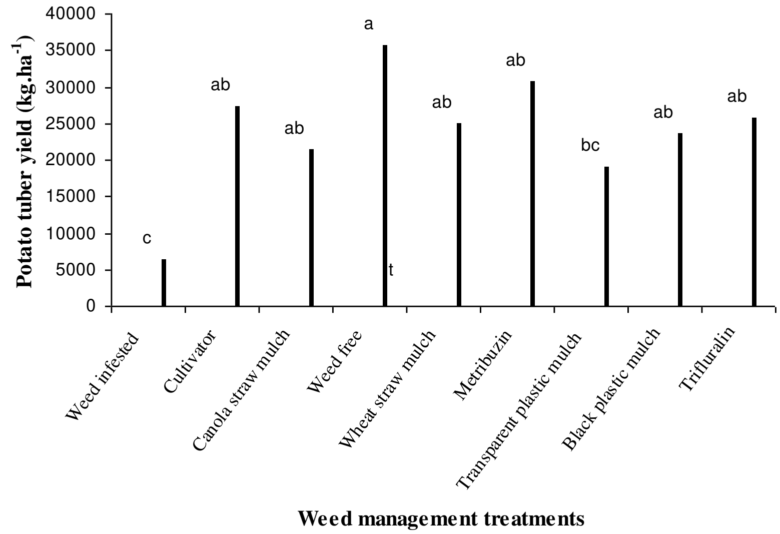

Figure 9. *The effect of different weed management treatments on tuber yield (kg.ha^{-1}) in potato cultivation (The averages of at least one similar letter are not significantly different at* ($P \leq 0.05$)*, using Duncan's multiple range test)*

In present experiment, the highest potato tuber yield was obtained at a rate of 36 ton/ha at weed free treatment that compared with the treatments of plant mulch, cultivator, herbicides and black plastic mulch were not significantly different but the transparent plastic mulch was significantly different compared to weed free treatment and transparent plastic mulch had the lowest tuber yield (19.3 ton/ha) after the weed infested treatment (6.6 ton/ha). The greatest reduction in tuber yield (about 83%) was related to weed infested treatment and then transparent plastic mulch (about 47%), and weed infested treatment was not significantly different with transparent plastic mulch treatment (*Fig. 9),* I seems that this is related to the lack of proper control of transparent plastic mulch to repress weeds in the experiment region in present study (Jafari et al., 2013; Majd et al., 2014).

Majd et al, (2014) applied also plastic mulch, herbicide and cultivator to control weeds and Mohammadduost Chamanabad et al, (2011) used herbal mulch, herbicide and cultivator to control weeds. However all control weed methods in present experiment were used simultaneously, so this experiment completes the results of others. In fact we were able to compare all the methods simultaneously and we developed other experiments mentioned above.

Conclusion

The use of non-chemical treatments of mulches and cultivator had a significant effect on potato weed density in both stations, so that the least amount of weed density was obtained by application of wheat straw mulch. However, the lowest weed biomass was observed in wheat and canola mulches treatments. It is also concluded that ecological treatments can control weeds as much as the herbicides. However, no significant difference between treatments of mulch and herbicides in terms of weeds biomass in present study, because

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mulches and cultivators can as well as herbicides prevent of germination, growth and development of weeds, both in the initial stage of weeds growth and in the subsequent stages, so it can be concluded that the use of mulches can be more costly than the use of herbicide due to labor costs and costs related to mulch preparation, By ignoring the cost, it can be noted that the ecological methods such as application of plant residues can be more environmental friendly. The presence of herbicides in the ecosystem is harmful for health humans and animals.

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BLOOD PROFILES AND HISTO-MORPHOMETRIC ANALYSIS OF THE GASTROINTESTINAL TRACTS OF ROSS 308 BROILER AND INDIGENOUS VENDA CHICKENS FED THE SAME DIET

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Abstract. The aim of this study was to assess the blood parameters and gut histology of broiler and indigenous chickens at different ages. A total of 120 Ross 308 broiler and Venda chickens were assigned in a complete randomized design to assess blood profiles and gut histomorphology in order to determine the immunity and digestive ability of the two breeds. Blood profiles and gut histology of two breeds at ages 42 and 90 days were determined. A measuring tape and RADWAG digital scale (Model PS 750/C/2) were used to measure the lengths and weights, respectively. At 42 days, platelets and serum creatinine were higher ($P<0.05$) for Ross 308 broiler than Venda chickens. Total protein was higher ($P<0.05$) in Venda than Ross 308 broiler chickens. The blood parameters measured at 90 days were higher ($P<0.05$) in Ross 308 broiler. Platelets were higher ($P<0.05$) in Venda than in Ross 308 broiler chickens. Weights and lengths of the gut were higher ($P<0.05$) in Ross 308 broiler than Venda chickens at both 42 and 90 days. Jejunum villus height was higher ($P<0.05$) in Ross 308 broiler than in Venda chickens aged 42 days. Crypt depth was higher ($P<0.05$) in the duodenum and ileum of Ross 308 broiler than in Venda chickens. The villus height/crypt depth ratios were higher ($P<0.05$) in Venda than Ross 308 broiler chickens at both 42 days. Age and breed affects blood profiles of broiler and indigenous chickens. The histomorphology of Ross 308 broiler was superior which reflects better absorption capabilities compared to Venda chickens.
Keywords: *haematology, weights, lengths, villi height, crypt depth*

Introduction

Chickens contribute a lot to social and economic well-being of most rural households of South Africa. The feed intake, digestibility, growth rate and overall productivity of broiler and indigenous chickens vary (Pauwels et al., 2015; Rondelli et al., 2003). Haematological and blood parameters of chickens are reliable indicators of their health status and productivity (Abdi-Hachesoo et al., 2011). Indigenous chickens which are mainly abundant in rural areas are known to be tolerant and resistant to diseases than their counterpart Ross 308 broiler chickens (Abdi-Hachesoo et al., 2013). It is, therefore, important to understand and determine the haematological indices of indigenous chickens in order to better interpret their health status and immune system capability to tolerate poultry

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diseases. The gastrointestinal tract (GIT) of the chicken and its gut state and condition are accountable for nutrient digestion, metabolism and absorption (Mabelebele et al., 2014). The GIT of fast growing Ross 308 broiler chickens develop and mature faster than those of the slow growing indigenous chickens (Jamroz, 2005). Ross 308 broiler chickens have been selected for high feed conversion ratio and growth rate. This has resulted in changes in the anatomy and function of the digestive system (Krás et al., 2013). The length and weight of the small intestines vary between the different species of birds (Hassouna et al., 2001).

Histologically, villus height and mucosa thickness are normally used as a good index for understanding the intestinal status which is directly linked to the absorptive functions (Incharoen, 2013). Morphology and histology of the gastrointestinal tract of Ross 308 broiler chickens were described and studied previously by Aitken (1958), Calhoun (1954) and Hassouna (2001). However, in more recent studies by Mabelebele et al. (2014), the digestive organ weights and lengths of Ross 308 broiler and indigenous Venda chickens at 90 days of age were compared. These authors observed that although broiler chickens at 90 days of age had heavier and longer digestive organs however, Venda chickens had lower gizzard pH, which might be related with better fibre digestion. As such, not much information on the blood profiles, morphology and histological differences between Ross 308 broiler and indigenous Venda chickens is available. Therefore, the objective of this study was to determine the differences between the blood profiles, morphometric and histological analyses of Ross 308 broiler and indigenous Venda chickens raised under the same conditions and fed a similar diet.

Materials and methods

Study site and experimental design

This study was conducted at the University of Limpopo Animal Unit. The study commenced with 60 Ross 308 broiler and 60 Venda chickens aged 1 day. Ross 308 broilers chickens were acquired from Lufafa hatchery, Tzaneen, South Africa while indigenous Venda chickens were obtained from the University of Limpopo hatchery. The chickens were kept for a period of 90 days on a commercial diet (*Table 1*). Feed and water were provided *ad libitum* and except for the first day when 24 hours of light was provided, 18 hours of lighting and 6 hours of darkness in every 24-hour period was maintained throughout the trial period. Chickens were housed in pens, assigned to a complete randomized design with each breed replicated five times with twelve birds per replicate. All managements of the birds were carried out with the strict accordance of the rules and regulations of the University of Limpopo Animal Ethics Committee with the following ethics approval number (TREC/12/2014:IR).

Table 1. *Ingredients and nutrient contents of diets fed at 0-42 and 43-90 days.*

Ingredients (g/kg)	0-42 days	43-90 days
Yellow maize	672.53	738.43
SBM	156.21	140.97
Canola (solvent-extracted)	50.00	10.00
Meat meal	87.31	79.39
Canola Oil	12.75	15.43

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Limestone	2.36	2.46
Dical Phos 18P/21Ca	0.01	0.01
Salt	0.597	0.72
Na bicarb	1.82	1.89
Vitamin/mineral premix†	2.00	2.00
Choline Cl 70%	1.28	1.16
L-lysine HCl 78.4	4.04	3.81
DL-methionine	1.81	1.68
L-threonine	1.98	1.68
Chemical composition (g/kg)		
Metabolisable energy (MJ/kg)	12.97	13.39
Crude protein	215.0	195.0
Crude fiber	24.47	22.2
Crude fat	45.5	47.9
Digestible arginine	11.1	9.84
Digestible lysine	11.5	10.2
Digestible methionine	4.7	4.3
Digestible methionine + cysteine	7.0	6.3
Digestible leucine	9.0	7.4
Digestible tryptophan	1.7	1.5
Calcium	8.7	7.8
Available phosphorus	4.35	3.9
Potassium	2.3	6.6
Chloride	1.6	2.3
Sodium	1.6	1.6
Choline (mg/kg)	1600	1500

†The active ingredients contained in the vitamin–mineral premix were as follows (per kg of diet): vitamin A – 12000 IU, vitamin D3 – 3500 IU, vitamin E – 30.0 mg, vitamin K3 – 2.0 mg, thiamine – 2 mg, riboflavin – 6 mg, pyridoxine – 5 mg, vitamin B12 – 0.02 mg, niacin – 50 mg, pantothenate – 12 mg, biotin 0Æ01 mg, folic acid – 2 mg, Fe – 60 mg, Zn – 60 mg, Mn – 80 mg, Cu – 8 mg, Se – 0Æ1 mg, Mo – 1 mg, Co – 0Æ3 mg, I –1

Haematological values

Two chickens per replicate were selected for slaughter at 42 and 90 days of age. Before slaughter, 3 ml of blood samples from both Ross 308 broiler and Venda chickens were collected from a jugular vein into two test tubes per bird, one with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and the other without EDTA. The blood collection tubes were kept in ice to avoid denaturing of protein, and plasma was stored at -20℃ until further analysis. Blood samples were analysed within 2 hours of collection for haematocrit (HCT), red blood cell (RBC), white blood cell (WBC) and haemoglobin (HBG) according to the methods of Dein (1984). Haemoglobin amount was measured using the Beckman Coulter ACT diff Haematology Analyzer (Beckman-Coulter, USA). Platelet counts were also measured. The HCT was measured as micro haematocrit with 75 × 16 mm capillary tubes and centrifuged at 2500 rpm for 5 minutes. Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) were calculated according to Ritchie et al. (1994). Serum parameters analysed included total protein, albumen, glucose, cholesterol, triglycerides, calcium, phosphorus, sodium and potassium (Aravind et al., 2003).

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Morphology of the gastrointestinal tracts

In order to evaluate organ weights and intestinal morphometrics of the chickens at 42 and 90 days of age, the gizzard, proventriculus, small intestines and the large intestines, including the caeca, were collected and weighed immediately at both 42 and 90 days. A measuring tape and RADWAG digital scale (Model PS 750/C/2) were used to measure the lengths and weights, respectively. The gizzard was weighed after the contents were removed and cleaned. After removal of the contents, the small and large intestines were cut in segments, cleaned, weighed and measured. The small intestines were measured from the site where the duodenum emerges from the gizzard and the beginning of the caeca, and the large intestine length included the length of the colon and the rectum. Chickens were weighed before slaughter.

Digestibility and Histological analysis

Nutrient digestibility at 42 days of age was determined according to the methods of McDonalds et al (1992). At the ages 42 and 90 days, 2 birds per pen were sacrificed by cervical dislocation. The whole small intestine was removed immediately and immersed in 10% buffered formalin. Three small intestine segments measuring approximately 3.0 cm of the duodenum (from the gizzard to pancreatic and bile duct), jejunum (from the bile duct to Meckel's diverticulum) and ileum (from the Meckel's diverticulum to ileo-caecalcolonic junction) were cut according to Samanya and Yamauchi (2002) for each chicken at ages 42 and 90 days. The samples were washed in phosphate buffer solution at 0.1 M (pH 7.4) and fixed in Bouin solution for three days. The samples were then trimmed to eliminate the torn edges, and remained for further 24 hours in the fixing solution. The samples were washed in ethanol at 70% to remove the fixing solutions, dehydrated in graded series of alcohol and cleaned in xylol. Four semi-serial sections with 7-cm thickness were placed in each slide. The slides were dyed using the method of the periodic acid of Schiff (PAS). Using an image capture and analysis system (Image J, 1.47r), villi height and crypt depths were measured in duodenum, jejunum and ileum sections were measured. Villus height was measured from the basal region, which starts at the higher portion of the crypt, until villus tip, whereas crypt depth was measure from the base up to the crypt-villi transition region (Carrijo et al., 2005).

Statistical analysis

Data on haematology, morphology and histology of the GIT of both Ross 308 broiler and Venda chickens were analysed using the General Linear Model (GML) procedure of the statistical analysis of variance (SAS, 2008). Differences were considered significant at $P<0.05$.

Results

Haematological and biochemical parameters of Ross 308 broiler and Venda chickens aged 42 and 90 days

The results of the blood profiles of Ross 308 broiler and Venda chickens aged 42 days are presented in *Table 2*. No significant ($P>0.05$) differences were observed on the complete blood count and biochemical parameters between the chicken breeds except for platelets and serum creatinine. The platelets were higher ($P<0.05$) in Ross 308 broiler than in Venda chickens. Serum creatinine at 42 days of age was higher ($P<0.05$)

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for Ross 308 broiler than Venda chickens. However, the total protein of Venda chickens at 42 days of age were higher (P<0.05) than those of Ross 308 broiler chickens. The results of the blood profiles of Ross 308 broiler and Venda chickens aged 90 days are presented in *Table 3*. The WBC, RBC, HGB, HCT and MCV were lower (P<0.05) in Venda than Ross 308 broiler chickens. However, the platelets were lower (P<0.05) in Ross 308 broiler than Venda chickens. No significant differences (P>0.05) in MCH, MCHC, RDW were observed between Ross 308 broiler and Venda chickens aged 90 days. Serum calcium, creatinine, cholesterol and albumen were higher (P<0.05) in broiler than indigenous chickens. However, sodium, potassium, phosphorus, glucose and total protein were similar in Ross 308 broiler and Venda chickens. Venda chickens had higher (P<0.05) serum triglycerides than those in Ross 308 broiler chickens.

Table 2. *The haemato-biochemical profiles of Ross 308 broiler and Venda chickens aged 42 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
WBC (× 10^3 cell/µL)	90	88	3.32
RBC (× 10^6 cell/µL)	2	2	0.27
Haemoglobin (g/dL)	10	13	1.26
Haematocrit (L/L)	32	39	3.95
MCV(fL)	134	137	4.98
MCH (pg)	45	45.	0.56
MCHC (g/dL)	33	32.	0.34
RDW %	7	7.	0.10
Platelets	59^a	24^b	10.13
Sodium (mmol/L)	162	156	5.21
Potassium (mmol/L)	10	10	0.64
Calcium (mmol/L)	2	2	0.07
Magnesium (mmol/L)	1	0.9	0.24
Glucose (mmol/L)	17	14	2.15
Creatinine(µmol/L)	17^a	9^b	5.05
Cholesterol (mmol/L)	3	4	0.66
Triglyceride (mmol/L)	0.3	0.3	0.16
Phosphorus (mmol/L)	6	7	0.64
Albumen (g/L)	3	2	3.20
Total protein (g/L)	20^b	25^a	0.15

SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Table 3. *The haemato-biochemical profiles of Ross 308 broiler and Venda chickens aged 90 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
WBC (× 10^3 cell/µL)	734^a	662.6^b	18.97
RBC (× 10^6 cell/µL)	2^a	1.8^b	0.15
Haemoglobin (g/dL)	10^b	12.6^a	0.66
Haematocrit (L/L)	0.2^a	0.2^b	0.01
MCV(fL)	$112.^a$	101.3^b	4.10
MCH (pg)	53	52.9	2.75
MCHC (g/dL)	47	46.8	3.11
RDW %	18	19.2	1.05
Platelets	12^b	14.4^a	3.29

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Sodium (mmol/L)	157	150.0	2.66
Potassium (mmol/L)	11	10.3	0.65
Calcium (mmol/L)	2 [a]	2.5 [b]	0.02
Glucose (mmol/L)	15	15.6	0.20
Creatinine(µmol/L)	10[a]	8.4 [b]	0.76
Cholesterol (mmol/L)	4[a]	3.3 [b]	0.12
Triglyceride (mmol/L)	0.1 [b]	0.3 [a]	0.03
Phosphorus (mmol/L)	2	2	0.05
K8Albumen (g/L)	10[a]	8[b]	0.23
Total protein (g/L)	42	38	1.81

SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Tables 4 and *5* show the live and carcass weights and gastrointestinal weights of Ross 308 broiler and Venda chickens aged 42 and 90 days. The live and carcass weights were heavier (P<0.05) for Ross 308 broiler than Venda chickens at both ages. The GIT weights of Ross 308 broiler were heavier (P<0.05) than those of the Venda chickens at both ages. The crop, proventriculus and gizzards were heavier (P<0.05) in Ross 308 broiler than in Venda chickens at both ages. Heavier weights (P<0.05) for small intestines, duodenum, jejunum, ileum, large intestines and the caeca were obtained in Ross 308 broiler than Venda chickens at both ages.

Table 4. *The gastrointestinal tract weights (g) of Ross 308 broiler and Venda chickens aged 42 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
Live-weight	1443[a]	520[b]	489.91
Carcass weight	1004[a]	309[b]	369.55
GIT	198[a]	89[b]	60.39
Crop	12[a]	4[b]	5.39
Proventriculus	7[a]	3[b]	2.17
Gizzard	38[a]	15[b]	12.29
Small intestine	50[a]	20[b]	16.38
Duodenum	30[a]	12[b]	9.78
Jejunum	9[a]	4[b]	3.07
Ileum	7[a]	3[b]	3.73
Large intestine	8[a]	4[b]	2.28
Caeca*	4[a]	2[b]	2.19

* An average value of each caeca pair
SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Table 5. *The gastrointestinal tract weights (g) of Ross 308 broiler and Venda chickens aged 90 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
Live-weight	2861[a]	1339[b]	102.47
Carcass weight	2105[a]	830[b]	82.54
GIT	200[a]	120[b]	9.41
Crop	16[a]	8[b]	1.13
Proventriculus	11[a]	7[b]	0.45
Gizzard	53[a]	36[b]	2.65
Small intestine	78[a]	47[b]	7.27

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Duodenum	23[a]	10[b]	4.66
Jejunum	25[a]	14[b]	1.71
Ileum	26[a]	21[b]	2.18
Large intestine	32[a]	12[b]	0.56
Caeca*	7[b]	10[a]	2.41

* An average value of each caeca pair
SEM: Standard error of the mean
[k8a,b] Means within the same row with different letters are significantly different (P<0.05)

The lengths of the gastrointestinal tract are presented in *Tables 6* and *7*. A similar trend observed for GIT weights was also observed for lengths with the entire tract being longer (P<0.05) for Ross 308 broiler than Venda chickens at 42 days of age. The small intestines, duodenum, ileum, large intestines and the caeca were shorter (P<0.05) for Venda than Ross 308 broiler chickens. At 90 days of age, the gastrointestinal lengths of the Venda chickens followed a similar tendency as their weights. The entire GIT length, small intestines and its segments, the large intestines and caeca were longer (P<0.05) for Ross 308 broiler than Venda chickens.

Table 6. *The gastrointestinal tract lengths (cm) of Ross 308 broiler and Venda chickens aged 42 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
GIT	202[a]	124[b]	42.42
Small intestine	147[a]	98[b]	29.49
Duodenum	95[a]	53[b]	22.80
Jejunum	39[a]	23[b]	7.82
Ileum	27[a]	19[b]	10.39
Large intestine	13[a]	11[b]	3.55
Caeca*	17[a]	9[b]	2.84

* An average value of each caeca pair
SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Table 7. *The gastrointestinal tract lengths (cm) of Ross 308 broiler and Venda chickens aged 90 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
GIT	208[a]	138[b]	8.96
Small intestine	179[a]	114[b]	8.86
Duodenum	34[a]	22[b]	1.92
Jejunum	64[a]	39[b]	2.27
Ileum	89[a]	57[b]	2.67
Large intestine	39[a]	17[b]	0.53
Caeca*	22[a]	11[b]	4.08

* An average value of each caeca pair
SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

The results of the villus height, crypt depth and villus/crypt depth ratio of small intestine segments between Ross 308 broiler and indigenous Venda chickens are presented in *Tables 8* and *9*. Similar villus heights (P>0.05) were observed on

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duodenum and ileum of Ross 308 broiler and Venda chickens aged 42 days. However, the jejunal villus height of Ross 308 broiler were higher (P<0.05) than those of Venda chickens aged 42 days. Duodenum and ileum crypt depths of Ross 308 broiler were higher (P<0.05) than those of Venda chickens aged 42 days. Similar (P>0.05) jejunum crypt depths were observed between Ross 308 broiler and Venda chickens. Venda chickens exhibited higher (P<0.05) villus/crypt depth ratios in all segments of the small intestines than Ross 308 broiler chickens at 42 days of age. Villus heights of the duodenum, jejunum and ileum were similar (P>0.05) between Ross 308 broiler and Venda chickens at 90 days. Crypt depths of the duodenum and ileum were higher in Ross 308 broiler chickens than in Venda chickens. However, no differences (P>0.05) in jejunal crypt depths were observed between the two breeds. Venda chickens had higher (P<0.05) ileum villus/crypt depth ratio than Ross 308 broiler chickens at ages 90days. No differences (P>0.05) were recorded on duodenum and jejunum villus/crypt depth ratios of Ross 308 broiler and Venda chickens aged 90 days. The results of nutrient digestibility of a grower diet fed to Ross 308 broiler and Venda chickens are presented in *Table 10*. Ross 308 broiler chickens showed higher (P<0.05) dry matter and crude protein digestibility, metabolisable energy and N retention than in Venda chickens.

Table 8. *Histological measurements of duodenal, jejunal and ileal wall of Ross 308 broilers and Venda chickens aged 42 days*

Parameters	Duodenum			Jejunum			Ileum		
	Ross 308 broiler	Venda chickens	SEM	Ross 308 broiler	Venda chickens	SEM	Ross 308 broiler	Venda chickens	SEM
Villus height (mm)	758	783	21.4	720^{a}	624^{b}	22.43	617	665	21.07
Crypt depth (mm)	187^{a}	130^{b}	9.80	173	166	38.17	145^{a}	104^{b}	4.64
Villus/Crypt ratio	4^{b}	6^{a}	0.269	3^{b}	6^{a}	0.42	4^{b}	6^{a}	0.31

SEM: Standard error of the mean
[a,b] Means within the same column with different letters are significantly different (P<0.05)

Table 9. *Histological measurements of duodenal, jejunal and ileal wall of Ross 308 broilers and Venda chickens aged 90 days*

Parameters	Duodenum			Jejunum			Ileum		
	Ross 308 broiler	Venda chickens	SEM	Ross 308 broiler	Venda chickens	SEM	Ross 308 broiler	Venda chickens	SEM
Villus height (mm)	1266	1259.	47.13	1146	1153	50.40	809	846	41.58
Crypt depth (mm)	339^{a}	275^{b}	11.54	302	253	24.19	319^{a}	219^{b}	35.19
Villus/Crypt ratio	3	4.1	0.28	4	4	0.35	2^{a}	4.^{b}	0.30

SEM: Standard error of the mean
[a,b] Means within the same column with different letters are significantly different (P<0.05)

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Table 10. *Nutrient digestibility of a grower diet fed to Ross 308 broiler and Venda chickens aged 42 days*

Parameters	Ross 308 broiler	Venda chickens	SEM
Digestibility (Decimal)			
DM	0.83[a]	0.56[b]	0.030
CP	0.92[a]	0.76[b]	0.071
ME*	15[a]	10[b]	0.263
N retention (g/bird/day)	6[a]	1[b]	0.041

DM: Dry matter
CP: Crude protein
ME: Metabolisable energy (MJ/kg DM)
SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Discussion

The blood parameters of chickens are regarded as dependable and good indicators of the bird's health status. Haematological indices are reported not to be consistent in chickens because of a number of reasons including diseases (Fudge, 2000b), stress (Bedonova et al., 2007), season (Hauptmanova et al., 2006) and genotype (Gayathri and Hegde et al., 2006). In the current study Ross 308 broiler and indigenous Venda chickens aged 42 days had similar haematological parameters. The values reported in the present study are within the normal ranges reported for Ross 308 broiler chickens aged 42 days by Tehrani et al. (2012) and Talebi et al. (2005). However, several studies conducted on indigenous chickens in other countries have reported contradicting haematological indices. Higher values of WBC (2.27 - 2.43 × 10^3 cell/µL) and MCH (66-70 pg) were observed in Nigerian local chickens (Peters et al., 2011) than the indigenous chickens in the current study. In the study by Pampori and Iqbal (2007), values of for HCT and Hb were higher in Kashmir native chickens than the values obtained in indigenous chickens in the present study. The platelets in the current study were higher in Ross 308 broiler than indigenous Venda chickens. The results were contrary to those reported by Maxwell et al. (1991) who showed lower thrombocyte (platelets) of Ross 308 broiler chickens at the age of 7 weeks. These cells help in the blood clotting process by promoting homeostasis (Paul et al., 2012) which, therefore, indicates that at 42 days of age in the current study the functioning of these cells were poor in Venda chickens as compared to Ross 308 broiler chickens.

At 90 days of age the WBC, RBC, HCT and MCV were higher for Ross 308 broiler than indigenous Venda chickens. Values in the current study were contrary described by Pampori and Iqbal (2007), Peters et al. (2011) and Albokhadiam et al. (2012) who found that indigenous chickens had higher haematological values or indices than broiler chickens. However, supporting the current findings is a report by El-Safty et al. (2006) which indicated that WBC and MCH were higher in broiler than indigenous chickens. The haemoglobin blood concentrations and platelets were higher for indigenous Venda chickens in the current study than Ross 308 broiler chickens. The higher haemoglobin values of indigenous Venda chickens in the present study are similar to those reported by Orawen and Aengwanich (2007) for indigenous chickens. However, MCH, MCHC and RDW were not influenced by breed in the present study.

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The serum parameters at the ages of 42 days were similar between the chicken breeds. However, the serum total protein was higher in indigenous Venda than in Ross 308 broiler chickens, the levels reported in the current study were still lower (20 g/L for Ross 308 and 25g/L for indigenous Venda chickens) than those reported (33-47g/L in Ross 308 broiler aged 42 days) in a study by Piotrowska et al. (2011). Total serum protein can extremely vary in chickens and its variation depends on internal and external factors and results from physiological roles in blood proteins. This parameter is important to measure the chickens' body condition. Other differences identified where serum creatinine was higher for Ross 308 broiler than indigenous Venda chickens at 42 and 90 days of age. Serum creatinine is an important indicator of protein metabolism, a by-product of phosphocreatine breakdown in skeletal muscles (Piotrowska et al., 2011). Its concentration is directly related to muscle mass and is associated with age and physical activity (Szabo et al., 2005; Rajman et al., 2006). The serum creatinine values for Naked neck and Kashmir indigenous chickens reported in the studies conducted by Peters et al. (2011) and Pampori and Iqbal (2007) were higher (13 and 14 µmol/L, respectively) than values of indigenous Venda chickens in the current study at 42 days of age. Lipid metabolites are associated with energy metabolism and reflect fluctuations occurring during the growth period of chickens. At 42 days of age there were differences observed in the triglycerides concentration between the breeds. However, high triglycerides concentrations levels in indigenous Venda chickens were observed at 90 days of age.

Higher serum triglycerides concentrations, most often, represent intensive lipid metabolism and transport. These results are supported by Piotrowska et al. (2011) who indicated that the serum triglycerides of Ross 308 broiler chicken decrease as they grow in age. However, on the contrary Abdi-Hachesoo et al. (2011) indicated that no serum triglycerides difference was observed between Iranian indigenous and Ross 308 broiler chickens. Higher serum cholesterol levels were observed for Ross 308 broiler in the current study. These findings are similar to those reported by Abdi-Hachesoo et al. (2011) and Simaraks et al. (2004) who showed that serum cholesterol of indigenous chickens is lower than other breeds. The authors went further to hypothesize that this might be because of the high body activity and high energy need in the indigenous chickens.

Minerals are essential for chickens' growth and are involved in many biosynthetic processes in the body. Calcium is mainly needed for ossification of bones, regulation of muscle activity and catalyzation of enzyme and hormonal synthesis (Underwood and Suttle, 1999). On the 90th day serum calcium was higher for Ross 308 broiler than indigenous Venda chickens. Abdi-Hachesoo et al. (2011) reported similar serum calcium values between Ross 308 broiler and Iranian indigenous chickens.

Differences in GIT weights and lengths between the two breeds used in the current study were observed. GIT were heavier and longer in Ross 308 broiler than indigenous Venda chickens. It was clearly evident in the present study that the slow growing indigenous Venda chickens abundant in rural South Africa and with lighter live and carcass weights, had inferior GIT weights and lengths. A study conducted by Borin et al. (2006) indicated that the GIT weights and lengths of indigenous chickens were heavier and longer compared to broiler chickens aged 120 days. The authors explained this by stating that due to the extensive traditional system these chickens are kept under the availability of bulky and fibrous feeds which will contribute to superior GIT weights and lengths than those of the fast growing broiler chickens. These findings are contrary

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to the current study. The current study is supported by Khempaka et al. (2009), De Verdal et al. (2010) and Mossammi et al. (2011) who indicated that GIT lengths and weights of broiler chickens are superior. A recent study by Mabelebele et al. (2014) conducted on indigenous Venda and broiler chickens noted that broiler chickens with their heavier body weight tended to have heavier and longer GIT and digestive organs, with the exception of the gizzard under an intensive system which supports the current findings.

The intestinal villus and crypt morphology are associated with intestine function and growth in chickens. According to Tivey and Smith (1989), changes in enterocytes development and villi structure determine the digestive and absorptive capacity of the small intestine. The small intestine histomorphology including villus height, crypt depth and their ratio are important pointers of gut health in chickens. Increased villus height and villus height to crypt depth ratio are directly correlated with an increased epithelial turnover and longer villi are associated with activated cell mitosis (Montangne et al., 2003). On the other hand, a decrease in villus to crypt ratio or lower crypt to villus ratio is indicative of a higher rate of enterocyte-cell migration from the crypt to the villus (Adibmoradi et al., 2006; Silva et al., 2009). This can lead to poor nutrient absorption, increased secretion in gastrointestinal tract and reduced performance. In the current study the villus height and crypt depth in the duodenum and ileum were higher for Ross 308 broiler than indigenous Venda chickens. The smallest depth of the crypts was observed from indigenous Venda chickens. Decreasing crypt depth in indigenous chickens might be explained by the fact that the crypt can be regarded as the villus factory and a large crypt indicates rapid tissue turnover and a high demand for new tissues (Choct, 2009). The increased villus height in the small intestines could be associated with higher absorptive intestinal surface (Loddi et al., 2004) which facilitates the nutrient absorption and hence, has a direct impact on growth performance. This indicates that Ross 308 broiler chickens with longer and heavier GIT had better absorptive functions and, therefore, growth as compared to indigenous Venda chickens. At 90 days of age, chickens had similar intestinal villus height. This, however, does not imply that the breeds had similar nutrient absorption potential as the crypt depths are different. Ross 308 broiler chickens had higher crypt depth which is indicative of a higher epithelial cell turn over to permit renewal of the villus as needed (Langhout et al., 1999; Sobayo et al., 2012). Thus, a better absorption potential is evidenced by Ross 308 broiler chickens compared to indigenous Venda chickens. The better intestinal and absorptive functions in Ross 308 broiler chickens, perhaps, can be best interpreted by higher nutrient digestibility and N retention as observed in the current study.

Conclusion and Recommendations

Age affects the blood profiles of both broiler and indigenous chickens. It was observed that at age 42 days, most haematological and biochemical parameters were similar between broiler and indigenous Venda chickens. However, platelets and serum creatinine were higher for Ross 308 broiler chickens at 42 days of age. At 90 days of age, WBC, RBC, Haematocrit, MCV, platelets, serum calcium, creatinine, cholesterol and albumen were higher for Ross 308 broiler chickens. Total protein at 42 days of age, platelets, haemoglobin, serum triglycerides at 90 days of age were higher for Venda chickens. It was expected that indigenous Venda chickens would have superior immunological abilities than broiler chickens in the current study. However, this was

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not the case, debunking the perception that indigenous chickens are hardier and more resistant to diseases than the broiler chickens. Perhaps the immunological abilities of indigenous chickens are superior when kept under extensive conditions, which was not the case in this study. A comparative study should be conducted on the blood profiles of indigenous chickens under intensive and extensive rearing systems.

Breed and age had an influence on the gastrointestinal tract lengths and weights. This was expected as broiler chickens have superior body weights with faster growth rate compared to indigenous chickens. The histology of the small intestine segments varied between breed and age. Higher villus height and deeper crypt depth were observed in Ross 308 broiler than indigenous chickens. This shows the better absorptive functions of the broiler chicken breed which in turn results to faster growth rate.

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BONE MORPHOMETRIC PARAMETERS OF THE TIBIA AND FEMUR OF INDIGENOUS AND BROILER CHICKENS REARED INTENSIVELY

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Abstract. Good structure and function of skeletal system are among the important factors in poultry management. Therefore, understanding the bone parameters of chickens is of paramount importance as they indicate the basic supportive, locomotive and protective functions. The aim of this study was to determine the bone measurements of Ross 308 broiler and Venda chickens aged 90 days. A 2 (breed) × 2 (sex) factorial arrangement in a completely randomised design was used and replicated four times with 20 birds per replicate. A total of 80 Ross 308 broilers and Venda chickens (20 males, 20 females of each breed) were sacrificed to measure the carcass, tibia, femur and fibula characteristics. A digital calliper and electronic scale were used to measure the length, diameter and weight of the bones. Ash contents of the bones were determined. Tibiotarsal, seedor and robusticity indices of the tibia and femur bones were measured. Tibia and femur lengths and widths were shorter in Venda chickens than those of Ross 308 broiler chickens. Ash contents of tibia and femur diaphysis and epiphysis of male and female Ross 308 broiler and Venda chickens varied significantly between the two breeds. Tibia and femur Seedor index were higher in males and females of Ross 308 broiler than Venda chickens. Diaphysis and medullary canal diameter were higher in Ross 308 broiler chickens than Venda chickens. The tibiotarsal index was lower for Ross 308 broiler than Venda chickens. Sex and breed affected the bone length, weight and width of Ross 308 broiler and Venda chickens at 90 days of age.
Keywords: *tibiotarsal index, robusticity, length, ash, seedor index*

Introduction

A fast growing broiler chicken is characterised by higher body weight gain, its skeleton (mainly femur and tibia bones) plays a major role as the supporting structure for its body weight (Applegate and Lilburn, 2002). Proper structure and function of skeletal system are among the important factors in animal management; information on bone morphometric will address this factor. According to Warris (2010), in the life cycle of animals, animal tissue development follows a precise order, the first being maturation of the nervous tissue followed by bone then muscle and lastly fat. Previous studies of Buckner et al. (1959) reported a relationship between body weight and various aspects of the tibia and femur bones in male and female New Hampshire chickens. Regression studies of their data designated that more than 98 % of the

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differences in tibia and femur length were a function of the body weight. Itoh and Hatano (1964) who specified that changes in femur ash and mineralisation were a reflective of the total skeletal mineralisation supported this. Study has shown that about 70 to 80% of bone mass is determined genetically, while 20 to 30% can be attributed to external factors in which management and diet are the most important factors (Eastell and Lambert, 2002). These factors also have significant effect on bone mineralization (Huyghebaert, 1997). Apart from the metabolic side-products of digestion and compounds synthesised in the intestine, vitamins, minerals or any other nutrient deficiency or excess are also among the important nutritional factors that may influence bone morphometric changes.

Commercial or intensive production systems (broiler chickens production system) are associated with temperature, photoperiod and light intensity controlled conditions, high energy diets, high plane of nutrition and high feed efficiency rates which encourage rapid growth and the early onset of the fattening phase (Lawrie and Ledward, 2006). Extensive production systems (Venda chicken production system), however, would be exposed to fluctuating temperatures and increased exercise on the forage area (Fanatico et al., 2005). Thus, it would be expected that the animals from the intensive production system would reach maturity at an earlier age and would produce heavier slaughter weight and carcasses with more fat if slaughtered at the same age as the free range animals. Castellini et al. (2002) reported lower growth rates and carcass weights of extensive birds compared to intensive birds. However, studies on morphology of the tibia and femur bones in indigenous chickens have not yet been reported. The densitometric and geometric parameters of the internal structures of the bones are of paramount importance as they indicate the basic supportive, locomotive and protective functions by the chickens (Charuta, 2013a). Bone mineral density in chickens is imperative as it helps to better understand and evaluate the process of bone mineral deposition (Louzada, 1994). Therefore, the objective of this study was to determine the bone characteristics of broiler and indigenous chickens aged 90 days.

Materials and methods

The University of Limpopo Animal Ethics Committee for the care and use of animals approved animal handling procedures for this experiment for scientific purposes with the approval number TREC/12/2014. The study was conducted at the University of Limpopo Experimental Farm, South Africa. The farm is situated 10 km North-west of the Turfloop campus of the University of Limpopo. The ambient temperature around the area is above 30°C during summer and below 25°C in winter. The farm lies at latitude 27.55 S and longitude 24.77 E and receives a mean annual rainfall of less than 400 mm. A total of 80-day-old broiler and indigenous chickens acquired from the University of Limpopo Experimental farm were used in this research. A 2 (breed) × 2 (sex) factorial arrangement in a completely randomised design was used and replicated four times with 20 birds per treatment. The chickens were raised under intensive system and provided with light; feed and water were offered *ad libitum* for the entire period of the experiment (90 days).

A total of 40 birds were slaughtered by cervical dislocation as recommended by the University of Limpopo Animal Science Ethics Committee. A total of 40 tibiotarsal and femoral bones from Ross 308 broiler and indigenous chickens (Ross 308 and Venda) (20 males and 20 females) were obtained and cleaned of the muscles by boiling them in

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deionized water for 10 minutes. For morphometric analysis, the two types of bones from each breed and sex were weighed (g) with a precision scale (RADWAG, Model PS 750/C/2). The length and width of the bones were determined using a measuring tape and a digital calliper (OMNI-TECH, SHA120). Tibia length was measured from the proximal end to the distal end and the width at the medial diaphysis. The widths of the proximal and distal tibia epiphyses were also measured. Femur length was measured from the proximal end of the shaft to lateral condyle and the width at the medial diaphysis (Zhang and Coon, 1997).

Geometric parameters (Tibiotarsal index, Seedor index and robusticity index) of the bones were determined and the thickness of the medial and lateral walls was measured at the midpoint mark using a dial caliper. Medullary canal diameter was calculated by subtracting the thicknesses of the medial and lateral walls from the diameter at the diaphysis while bone weight/length index also known as the Seedor index was obtained by dividing the tibia weight by its length (Seedor et al., 1991). The tibiotarsal and the robusticity indexes were determined using the following formulas (Reisenfeld, 1972), respectively:

$$\text{Tibiotarsal index} = \text{diaphysis diameter} - \text{medullary canal} \frac{\text{diameter}}{\text{diaphysis}} \text{diameter} \times 100 \quad \text{(Eq. 1)}$$

$$\text{Robusticity index} = \text{bone length}/\text{cube root of bone weight} \quad \text{(Eq. 2)}$$

The cleaned bones were taken to Polokwane Veterinary Clinic for X-rays analysis. Bones were oven-dried at 105°C for 24 h, ashed in a muffle furnace at 600°C for 6h, and ashed at 600° C overnight, cooled in a desiccator, and weighed. The samples were then ashed in a muffle furnace at 600°C for 24 h in crucibles (AOAC, 2000). All data was analysed using the General Linear Model procedure of the statistical analysis of systems (SAS, 2008). Analysis of variance was performed and the Duncan's Multiple Range tests were used for mean separation. The level of significance was set at P-value ≤ 0.05. Polynomials of the tibia and femur weights, lengths and widths as a function of the body weight were determined using the PROC REG procedure of statistical analysis of systems (SAS, 2008).

Results

The bone traits of male and female Ross 308 broiler and indigenous Venda chickens are presented in *Table 1* and *Figure 1*. Tibia, femur and fibula lengths were longer (P<0.05) for female Ross 308 broiler than the female Venda chickens. Similarly, male Ross 308 broiler had longer (P<0.05) tibia and femur bones than male Venda chickens. However, the fibula bones were similar (P>0.05) for male Ross 308 broiler and Venda chickens. The tibia and femur weights were heavier (P<0.05) for both male and female Ross 308 broiler compared to those of Venda chickens. Similar fibula weights (P>0.05) were observed for both male and female Ross 308 broiler and Venda chickens. The width of the tibia and femur followed the same trend as observed for the length and weight, with male and female Ross 308 broiler chickens having wider (P<0.05) bones than those observed in male and female Venda chickens. The fibula bone width, however, was not influenced (P>0.05) by either sex or breed of the chickens.

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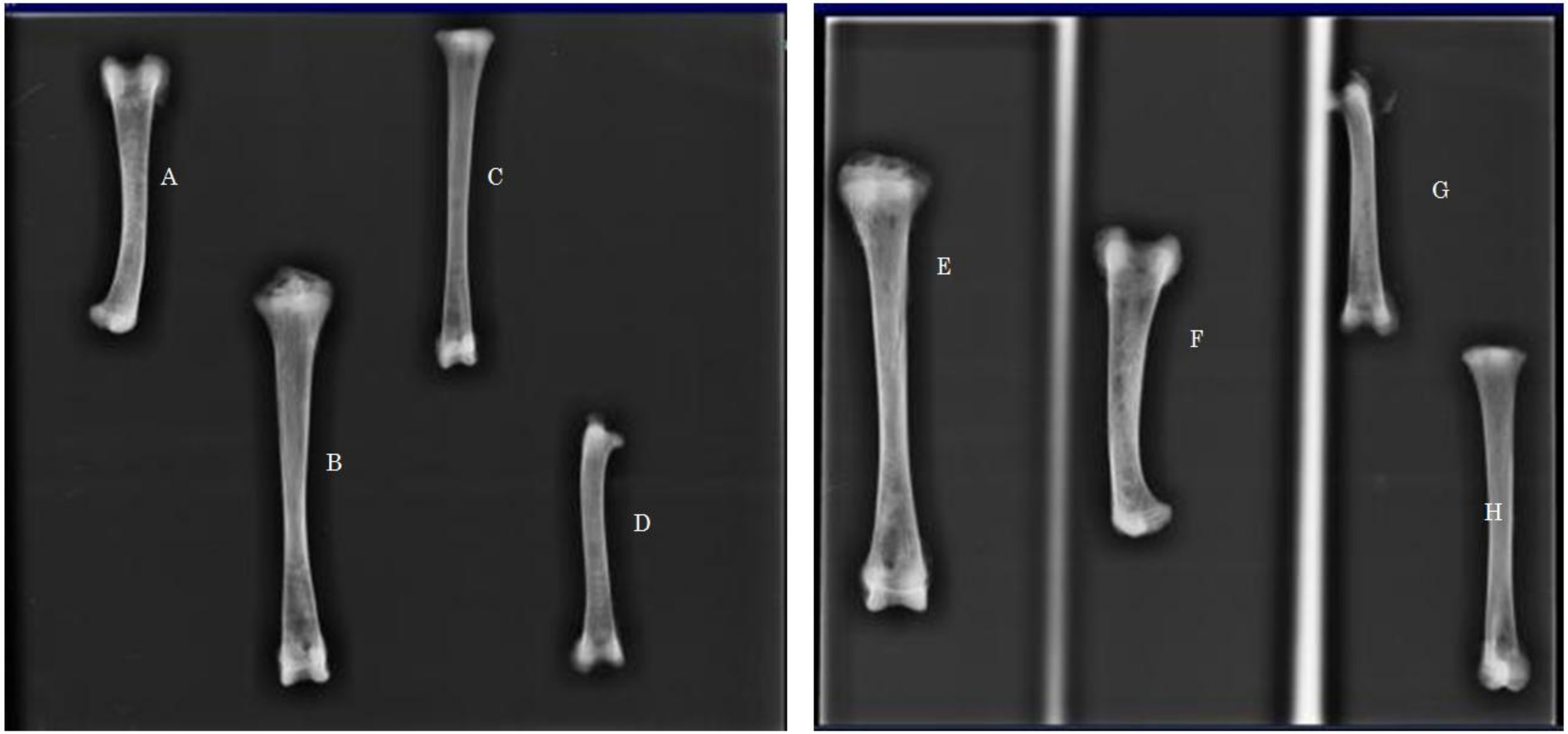

Figure 1. *A) Femur for male Ross 308 broiler chicken. B) Tibia for male Ross 308 broiler chicken. C) Tibia for male Venda chicken. D) Femur for male Venda chicken. E) Tibia for female Ross 308 broiler chicken. F) Femur for female Ross 308 broiler chicken. G) Femur for female Venda chicken. H) Tibia for female Venda chicken.*

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Table 1. *Bone length (mm), weight (g) and width (mm) of female and male Ross 308 broiler and indigenous Venda chickens*

Variable	Breed			
	Sex	**Ross 308**	**Venda**	**SEM**
Tibia length	Female	126.06[a]	115.52[b]	6.298
	Male	144.90[a]	117.41[b]	2.81
Femur length	Female	92.38[a]	82.44[b]	5.872
	Male	96.89[a]	85.84[b]	1.432
Fibula length	Female	89.70[a]	80.75[b]	1.450
	Male	86.42	80.87	5.904
Tibia weight	Female	25.58[a]	13.50[b]	1.085
	Male	36.91[a]	16.69[b]	1.283
Femur weight	Female	18.08[a]	11.14[b]	0.648
	Male	24.30[a]	14.75[b]	1.184
Fibula weight	Female	2.10	1.64	0.183
	Male	2.86	1.86	0.319
Body weight	Female	1862.01[a]	750.17[b]	37.538
	Male	2349.60[a]	890.20[b]	93.757
Tibia width	Female	8.79[a]	6.74[b]	0.295
	Male	10.19[a]	7.40[b]	0.445
Femur width	Female	9.42[a]	7.83[b]	0.305
	Male	10.85[a]	8.70[b]	0.206
Fibula width	Female	2.82	2.36	0.382
	Male	3.30	2.39	0.293

SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different ($P<0.05$)

The polynomial regression of tibia and femur length and weight of Ross 308 broiler and indigenous Venda chickens as a function of carcass weight are presented in *Figure 2*. Calculated as a function of carcass weight, 97 % of tibia length and 94 % of femur length in Ross 308 broiler chickens are a result of changes in body weight (*Figure 2A*). However, compared to Venda chickens when polynomial functions were calculated, lower values of 89 % of tibia length and 37 % of femur length were because of variations in carcass weights (*Figure 2C*). When bone weight was determined as a function of carcass weight, 95 % and 88 % of tibia weight of Ross 308 broiler and Venda chickens were observed, respectively. Similar values of 93 % and 91 % of femur weight of Ross 308 broiler and Venda chickens were observed as function of carcass weight, respectively (*Figures 2B* and *2D*).

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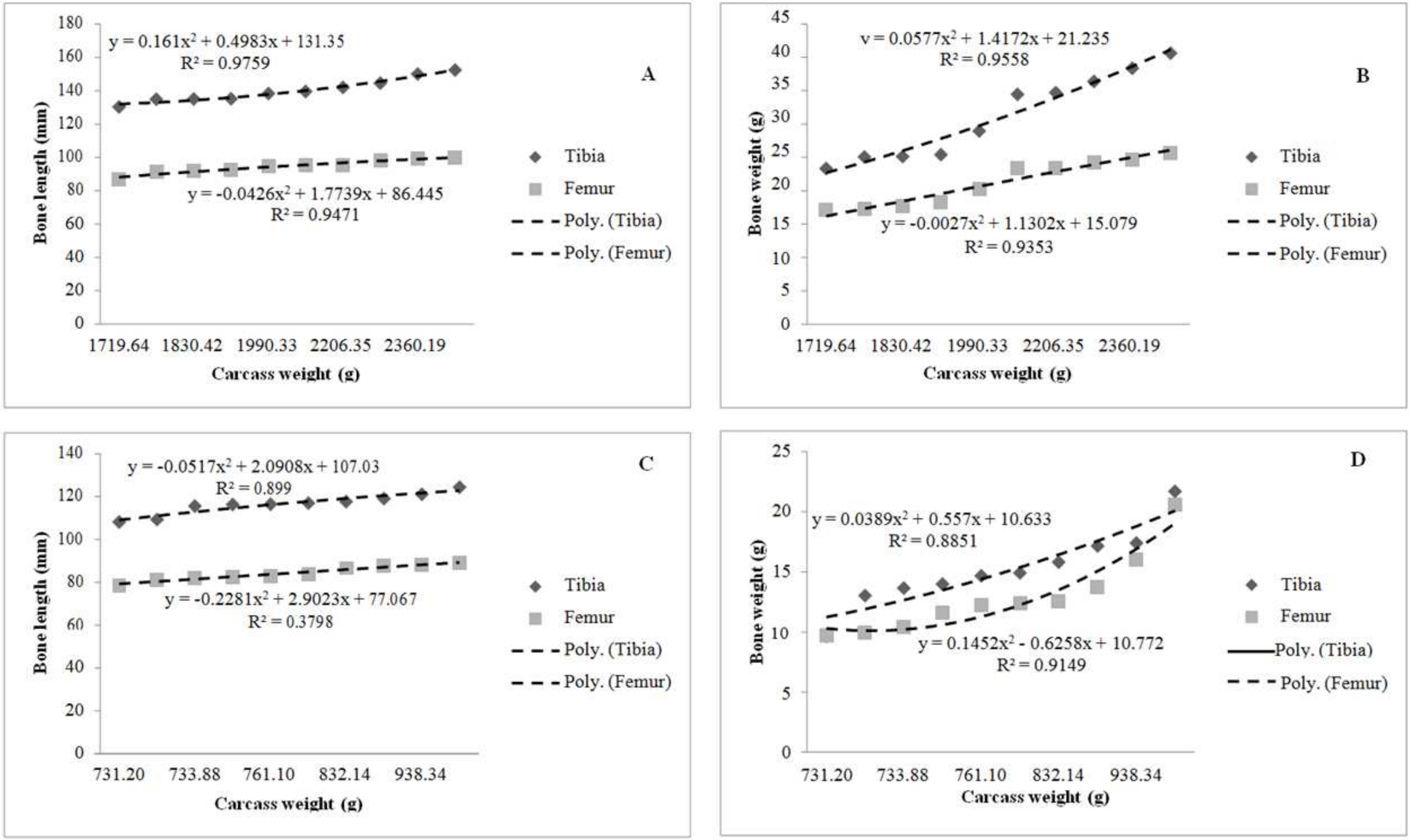

Figure 2. *A) Regression of Ross 308 broiler tibia and femur length (mm) on carcass weight. B) Regression of Ross 308 broiler tibia and femur weight (g) on carcass weight. C) Regression of indigenous Venda chickens' tibia and femur length (mm) on carcass weight. D) Regression of indigenous Venda chickens' tibia and femur weight (g) on carcass weight.*

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Tibia and femur geometric parameters of male and female Ross 308 broiler and indigenous Venda chickens are shown in *Table 2*. Tibia Seedor index was higher (P<0.05) in males and females of Ross 308 broiler than their counterparts Venda chickens. The femur Seedor index also followed the similar trend as tibia Seedor index with higher values (P<0.05) reported for male and female of Ross 308 broiler than Venda chickens. No differences (P>0.05) were observed for tibia and femur robusticity indices of male and female Ross 308 broiler and Venda chickens. The tibia diaphysis and medullary canal diameter of male and female Ross 308 broiler chickens were higher (P<0.05) than those in Venda chickens. The tibiotarsal index was higher (P<0.05) for male and female Venda chickens than for Ross 308 broiler chickens.

Table 2. *Bone geometric analysis of male and female Ross 308 broiler and indigenous Venda chickens*

Variables	Breed			
	Sex	**Ross 308**	**Venda**	**SEM**
Tibia Seedor Index(mg/mm)	Female	207.81[a]	116.31[b]	14.765
	Male	254.84[a]	141.49[b]	7.953
Femur Seedor Index(mg/mm)	Female	196.58[a]	135.68[b]	9.810
	Male	251.05[a]	175.57[b]	15.191
Tibia Robusticity Index	Female	4.28	4.88	0.219
	Male	4.35	4.61	0.064
Femur Robusticity Index	Female	3.51	3.70	0.104
	Male	3.34	3.53	0.109
Diaphysis Diameter (mm)	Female	9.62[a]	7.80[b]	0.420
	Male	10.38[a]	8.63[b]	0.398
Medullary canal diameter (mm)	Female	6.93[a]	5.47[b]	0.162
	Male	7.31[a]	5.99[b]	0.344
Tibiotarsal Index	Female	27.37[b]	33.50[a]	0.469
	Male	25.00[b]	32.35[a]	0.938

SEM: Standard error of the mean
[a,b] Means within the same row with different letters are significantly different (P<0.05)

Ash contents of tibia and femur diaphysis and epiphysis of Ross 308 broiler and indigenous Venda chickens are presented in *Figures 3*. Ash content of the femur epiphysis was higher (P<0.05) in Venda male than the male of Ross 308 broiler chickens. However, female Ross 308 broiler and Venda female chickens exhibited similar (P>0.05) femur epiphysis ash contents. Tibia epiphysis ash contents were not different (P>0.05) between males and females of Ross 308 broiler and Venda chickens. Tibia diaphysis ash content was higher (P<0.05) in Venda males than Ross 308 broiler males. Similar tibia diaphysis ash content (P>0.05) were observed in the females of Venda and Ross 308 broiler chickens. A higher (P<0.05) femur diaphysis ash content was observed in Venda females than males of Ross 308 broiler and Venda chickens. Ross 308 broiler male had lower (P>0.05) femur diaphysis ash content.

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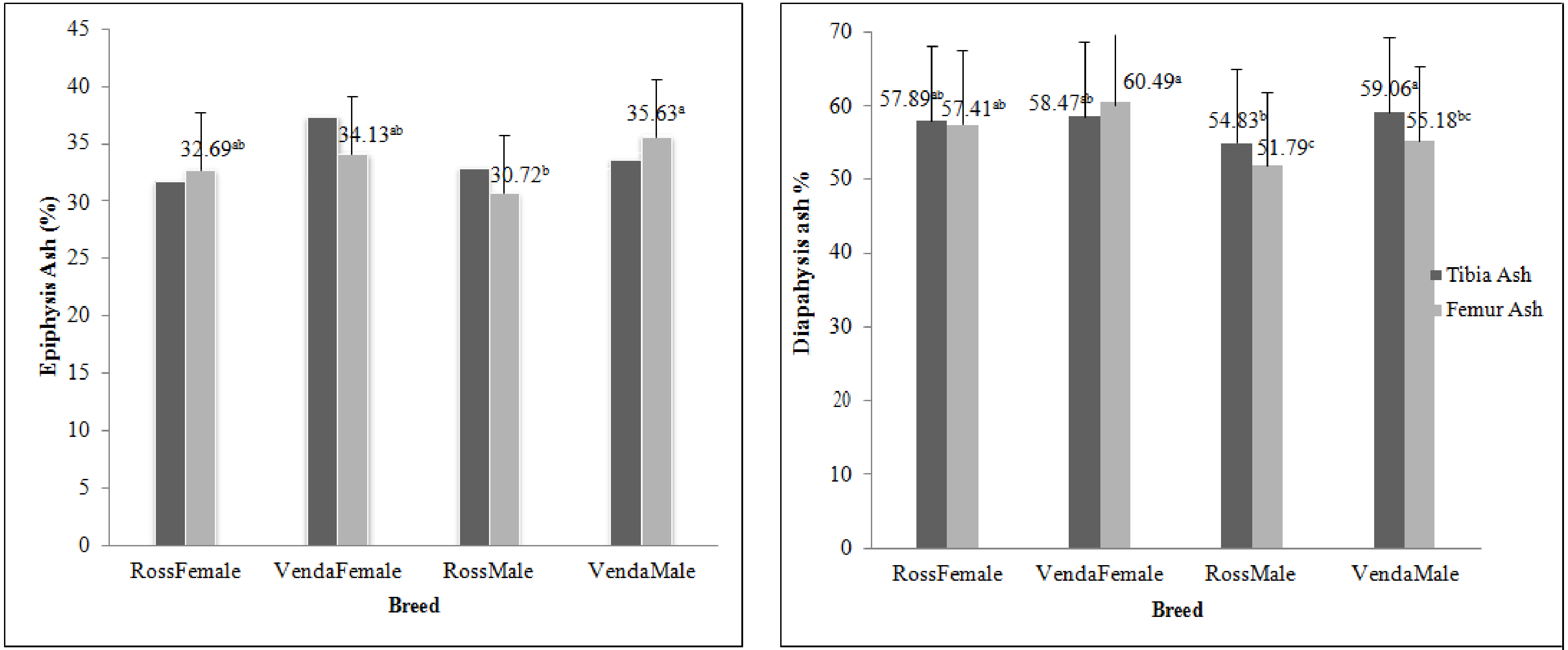

Figure 3. *Tibia and Femur Epiphysis and Diaphysis Ash (%) of Female and Male Ross 308 and Indigenous Venda aged 90 days.*
[a, b ,c]Means within the same column with different letters are significantly different (P<0.05).

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Discussion

In recent decades, selection pressure has been applied in poultry for fast growth and high meat production (Breugelmans et al., 2007; Kirkwood et al., 1989). This, in turn, puts pressure on the skeleton of the bird to support the heavier body weights associated with broiler chickens (Breugelmans et al., 2007). The femur bone length in the current study at 90 days for male and female Ross 308 broiler were 96.89 and 92.38 mm, respectively, whereas for male and female Venda chickens they were 85.44 and 82.44 mm, respectively. The values in the present study were lower than those reported by Breugelmans et al. (2007) who indicated that at 98 days of age Ross 308 broiler chickens had a femoral length of 116 mm, this might be due to age differences. The tibia lengths for Ross 308 broiler were 144.90 for males and 126.06 mm for females in the current study. This is similar to the previous results obtained by Latimer (1927) reported similar results for broiler chickens to those observed in the current study. The tibia lengths of male and female Venda chickens were 117.4 and 115.5mm, respectively. These values are lower compared to those of male and female Ross 308 broiler chickens at the same age in the current study. This can be attributed to breed differences. The males in the present study, regardless of the breed, had longer tibia and femur lengths compared to their females. Charuta et al. (2013a) and Rose et al. (1996) who indicated that bone length is related to sexual dimorphism support these results. The lengths of both the tibia and femur of slow growing Venda chickens, irrespective of the sex, were lower compared to those of the fast growing broiler chickens. Therefore, breed had an effect on the bone length of chickens.

Calculated as a function of carcass weight, tibia and femur lengths were compared and R-value of 97 % was a result of the variability in carcass weight of Ross 308 broiler chickens; however, only 89 % was recorded for indigenous Venda chickens. Buckner et al. (1950) observed a > 98 % as differences in tibia bone lengths in male and female New Hampshire chickens aged 196 days. The bone weights of chickens breeds used in this study were significantly higher for males than females. This was similar to the findings of Applegate and Lilburn (2002) who observed that sex had an effect on tibia and femur weights. However, slow growing Venda chickens in the current study with lighter body weight had lower tibia and femur weights compared to their counterparts Ross 308 broiler chickens. Although when the polynomial function of tibia and femur weights was calculated, R-value of 88 % for tibia and 91 % for femur are results of variations of the body weight for indigenous Venda chickens. Applegate and Lilburn (2002) and Buckner et al. (1950) observed that > 98 % of the variations in tibia and femur weights are function of the body weight for broiler chickens aged 43 days and New Hampshire chickens aged 23 weeks. Tibia and femur width of the male and female Ross 308 in the current study tended to follow a similar trend as the bone weights and lengths. Increase in bone length would be expected to correlate with the bone width indicating the overall bone size (Van Wyhe et al., 2012) which was the case with Ross 308 broiler chickens in this study. If the bone length continues to grow and increase without the increase in bone width, this could predispose chickens to increased skeletal problems.

Epiphyseal ash content of the femur was higher for Venda male chickens in the current study. The epiphyseal region of the bone is responsible for the linear increase in bone growth and this process amongst others includes mineralization (Van Wyhe et al., 2012; Applegate and Lilburn, 2002). The percentage of ash in this region would be expected to vary according to nutrition and age. These results are supported by Lin et al.

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(2012) indicating that bone ash was higher for Taiwan male chickens at 14 weeks of age. The reasons why both male and female indigenous Venda chickens had higher epiphyseal ash content compared to their counterparts is unclear in the current study. Ash content was higher in the tibia and femur diaphyseal region of male and female indigenous Venda chickens compared to lower percentile values in male and female of Ross 308 broiler chickens. Thorp (1992) compared the ash content at the femur diaphysis with the tibia one and observed that ash content was lower in femur, suggesting that this bone can be a vulnerable point of chickens and can be responsible for long bone abnormalities. Previous studies by Itoh and Hatano (1964); Dilworth and Day (1965) and Moran Junior and Todd (1994) concluded that femur bones in Ross 308 broiler chickens are more sensible to changes in diet than tibia bone.

The Seedor index that is also known as bone weight/bone length index is an indication of bone density (Seedor, 1995; Ameida and Bruno, 2006). Higher bone densities in the current study were observed for male and female Ross 308 broiler chickens than Venda chickens. These higher densities in Ross 308 broiler chickens are an indication of their heavier weight. A low robusticity index is an indication of strong bone structure (Safaeikatouli et al., 2012). Robusticity indexes of both tibia and neither breed nor sex affected femur bones. This was not expected for Venda chickens, as indigenous chickens are known to have stronger bone structure than broiler chickens. Perhaps, the reasons why indigenous chickens in the current study had similar robusticity index with broiler chickens would be the similar rearing systems under which both breeds were reared. Diaphysis and medullary canal diameter of Ross 308 broiler chickens were observed to be higher than in Venda chickens. Tibiotarsal index points out mineralization in the bone, and therefore a higher index indicates higher mineralization level in the bone (Ziaie et al., 2011).

Male and female Venda chickens had higher tibiotarsal index than male and female of Ross 308 broiler chickens. The above findings are supported and confirmed by the higher epiphyseal and diaphyseal ash content of male and female of Venda chickens in the current study. The higher mineralization in male and female Venda chickens is an indication of better bone quality than Ross 308 broiler chickens. Sex and breed affected the bone length, weight and width of Ross 308 broiler and indigenous chickens at 90 days of age. Bone geometry and densitometry analysis varied amongst breed and sex of chickens. Venda chickens had higher epiphyseal and diaphyseal ash content, which was confirmed by the higher tibiotarsal index. On the contrary, Ross 308 broiler chickens had higher femur and tibia Seedor index, which is an indication of denser bones. Although it was expected that indigenous Venda chickens that are hardier would have stronger bone structure than broiler chickens, this was not the case in this study. Perhaps the rearing system could have contributed to similar robusticity indexes that are an indicative of bone strength. This information will have implications when selecting chickens for better bone quality and strength.

Conclusion

In most of the morphometric parameters measured, both the male and female Ross 308 broiler chickens have superior values than the Venda chickens. However, in the ash contents, the male and female Venda chickens had higher values. Tibia and femur lengths were longer for female Ross 308 broiler than the female Venda chickens. Similarly, the tibia and femur weights were heavier for both male and female Ross 308 broiler compared to those of Venda chickens. However, fibula weights were similar for both male and female Ross 308 broiler and Venda chickens. The width of the tibia and femur followed the same trend as observed for the length and weight. Tibia and femur Seedor index were higher in males and females of Ross 308 broiler than the Venda chickens. The tibia diaphysis and medullary canal diameter of male and female Ross 308 broiler chickens were higher than those in Venda chickens. However, the tibiotarsal index was higher for male and female Venda chickens than for Ross 308 broiler chickens. Ash contents of the femur epiphysis and femur and tibia diaphysis were higher in Venda male than the male of Ross 308 broiler chickens. Similarly, tibia diaphysis ash content was higher in Venda males than Ross 308 broiler males. However, tibia and femur epiphysis ash contents were similar in both males and females of Ross 308 broiler and Venda chickens. The tibia diaphysis ash content was also similar in the females of Venda and Ross 308 broiler chickens.

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SPOREFORMING BACILLUS BIOEFFECTORS FOR HEALTHIER FRUIT QUALITY OF TOMATO IN POTS AND FIELD

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Abstract. Biologically active compounds, sugars, acids and antioxidants are key-important ingredients of healthy tomato food. Objective of the study is to investigate if specific industrial bioeffector products are improving the taste and some quality parameters of tomato fruits and how those results are appearing in pots and among organic field conditions? Spore-forming industrial *Bacillus amyloliquefaciens* FZB42 (RhizoVital) as single inoculums and combinations either with other *Bacillus* strains (Biorex-1), or with N_2-fixing-siderophore-producing strains (Biorex-2) were applied on *Solanum lycopersicon* Mill. var. Mobil test plant. Soil microbial counts, phosphorus availability and fruit quality, such as total soluble solids (TSS), content of some essential organic acids (citric-, maleic) and sugars (glucose, fructose) were assessed. The results found, that single industrial inoculums of FZB42 product had positive effect on P-availability and fruit quality in the pots, combinations of other biofertilizers, however, did not give additional results. Fruit quality parameters, TSS content, soluble sugars and organic acids were significantly improved ($p<0.05$) at both experimental conditions. Such better fruit taste was correlated significantly by the most probable number (MPN) total microbial counts with greater positive values in pot experiment, compared with more variable environmental field condition. The recommendation of using such bioeffector products is supported by the positive interrelation among measured soil characteristics and inside healthy quality parameters of organically grown tomato fruits.
Keywords: *bioeffectors, biofertilizers, phosphate solubilization, tomato quality, organic agriculture, MPN counts, correlation*

Introduction

Sustainable agriculture and healthy food become one of the most important aspects of any food-production systems. An increasing demand exists for fruits and vegetables of originating from ecological farming systems, avoiding the use of chemical fertilizers and pesticides (FIBL 2001; Hesammi et al., 2014; Kocsis et al., 2017). As a direct consequence of intensive agricultural production, deleterious environmental effects on soil-quality and fertility are increasing in parallel loss of the abundance and diversity of living organisms in soil-food-web (Johnston, 1986; Varga et al., 2007). Key important requirements therefore is the needs of reducing the artificial deleterious chemicals and xenobiotic inputs, more particularly among the agri- and horticultural practices (Juhos et al., 2015, 2016). Altogether with those conditions consumers' highlighted expectation for healthy and chemical-free foods are simultaneously required.

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Depending on the variability of environmental conditions 3-15 t microorganisms can be found in each hectare of the soils, not considering other soil-fauna elements of soil-food-web (Fekete et al., 2008; Kotroczó et al., 2008; Veres et al., 2013). For a correct assessment of soil edaphon, several parameters are investigated and variability of methods used, as a function of the aim and applicability of investigations. It is generally accepted, however that biological activity of soil organisms might play a crucial role in mobilization of soil nutrients and make them available and usable for higher plants, crops (Juhos, 2014). Several organisms are known to be involved in soil organic matter degradation and decomposition (Kotroczó et al., 2014a, b; Fekete et al., 2014, 2017; Veres et al., 2015) and giving with this process continuously the available nutrients for higher plants (Tóth et al., 2013). Various microbial diversity and types of microorganisms are living in the soils, including both of bacteria and fungi with known general and/or highly specific nutrient mobilization ability (Schweitzer et al., 2008). In recent decades' chemical fertilization was the most dominant in intensive agricultural production. By contrast active microbial soil characteristics were largely ignored, due to the mainly artificial plant nutrient fertilizing practices among the intensive agriculture production.

Concern for the above mentioned reasons for healthy environment, eco-friendly production methods and chemical-free farming systems are increasing (den Hollander et al., 2007). One of the most accepted and followed alternatives of intensive agricultural practices are the application of living bioeffectors (BE), which might include one or more types and strains of so called "beneficial microorganisms" in many soil-plant systems. Other, so-called non-living bioeffector materials (carriers, soil-ameliorative compounds, natural minerals) can be used in parallel with the generally accepted biofertilizer, bioeffector treatments. Such combined application is providing both living organisms and also soil-improving materials; finally, the survival of microbes and soil functions might be enhanced in one step. The selected beneficial microbes are known to support plant-growth and development, nutrient and water uptake of plants and might suppress damage of harmful soil-borne plant pathogen microorganisms in soil-plant systems. Bioeffectors can successfully integrate into any environmental-friendly agri-horti-, silvi- and viti-cultural practices. Main accepted application of bioeffectors is reducing or diminish fertilizer and high levels of pesticide inputs. Among beneficial microorganisms, the symbiotic connection between plant growth promoting bacteria (PGPB) or -rhizobacteria (PGPR) and their host plants was first described and demonstrated by Kloepper and Schroth (1978). PGPB or PGPR microorganisms have a potentially great impact on plant growth, yield and health with several known mechanisms in soil-plant systems. Those organisms might protect cultivated plants from soil-borne plant pathogens in addition they can improve soil chemical and physical characteristics (Glick et al., 2007). By using PGPB microorganisms as introduced inoculums of soils, nitrogen and phosphorus content, other mezo-micro-elements become available by plants. This mechanism can result harmonized nutrient balance in soils and might heal nutrient disorders in soils. As a direct consequence of inoculation, amount of synthetic fertilizers, furthermore other xenobiotics, toxic agrochemicals will be reduced (Hay et al., 2010).

Direct and indirect beneficial effects and mechanisms of bioeffectors are reported from any soil-plant-microbe systems, with known distinct and well-characterized effects. The combination of those described mechanisms are also possible, and different bacteria or fungal species might have more than one of these functional traits. Schippers

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et al. (1985) described three different affecting mechanisms in plant-soil-microbe systems:

- increased nutrient supply, nitrogen fixation, nutrient exploration or nutrient transport,
- increased defence mechanisms against some soil-borne plant pathogen fungi and bacteria by inhibiting of their growth; competition for space and for nutrients, antibiosis, parasitism, induced systemic resistance;
- direct enhancement of plant growth, production of several hormone-like, plant growth regulating (PGR) substances.

These protected effects can overlap in many cases and different microorganisms can also have more than one efficient PGPR or plant growth regulating (PGR), hormone-like mechanisms. In several occasions not all of these effects might be developed, due to the fact, that they might be modified during the vegetation periods through the various biotic and abiotic environmental (stress) factors (Biró et al., 2000; Carvalhais et al., 2013). Those particular effects are especially specific for combined "second-generation" of microbial inoculums, where microorganisms as specifically adapted ones were used to certain environment and for certain host plants. Specific bioeffective and stress-tolerant microbes therefore are used efficiently at the practice of amelioration, recultivation and/or at remediation practices (Biró et al., 2000; 2012).

Phosphorus supply is key issue for optimal tomato production. Necessity of P-elements are especially crucial at early stage of plant growth. Presence of phosphorus-mobilizing microorganisms as *Bacillus* spp. bacteria, furthermore the *fluorescens* and *putida* types of *Pseudomonas* spp., which can influence indirectly the uptake of this element is also essential (Khan et al., 2009). Those microorganisms are able to mobilize hardly available phosphorus forms (Bashan et al., 2013) e.g. they can solubilize natural rock phosphates to be available for plants, improving the growth of roots, shoots and plant biomass or fruits. Due to those beneficial properties, bioeffector organisms are successfully involved in ecological farming practices (Hariprasad and Niranjana, 2009; Biró et al., 2012). Among bioeffector (BE) strains, those with spore-forming abilities are more tolerant and survivor at serious environmental stress conditions, therefore their application is highly expanded in industrial microbial inoculum productions (Hartmann et al., 2009).

The *Bacillus amyloliquefaciens* is a non-pathogenic soil bacterium. Similar to other *Bacillus* species it is capable of producing endospores allowing it to survive for extended periods of time. The species also shows some antifungal properties which are influenced by several environmental factors, including nitrogen availability (Caldeira et al., 2008; Choudry et al., 2015). *Bacillus* spp., i.e. *B. subtilis* is known as efficient in cellulose degradation, amylase production and can block phytopathogenic micro-organisms (Yang et al., 2009). *B. thüringiensis* is generally used against soil pests, by creating an alkaline reaction and leading to the serious pest destruction. *B. megaterium* is efficient in phosphate mobilization, the production in growth substances and vitamin B12 and it is also possible to modify the plant residues into humus.

Among beneficial microorganisms the nitrogen(N_2)-fixing bacteria are having the uppermost importance, including as free-living, associative and obligate symbiont forms in soil-plant systems. *Azotobacter chroococcum* bacteria are free-living nitrogen fixing microbes, generally with drought and cold tolerance (about 3-4 °C of soil temperature) ability *Azospirillum lipoferum* is an associative nitrogen fixing bacteria, which secondary interact with the roots of monocots and also known as auxins, gibberellins, cytokinin plant

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hormone producer (Biró et al., 2000; Diamantidis et al., 2000). *Pseudomonas putida* as other frequently used biofertilizer species, might produce siderophore-like compounds, efficiently using of iron ions from soil and it creates also some competitive inhibition towards soil-borne plant-pathogen organisms and in addition enhance plant-uptake of macro-, mezo-and micro-nutrients (Timmis, 2002). Among ecological farming production systems, the use of bioeffector treatments, including the biofertilizer and/or the biopesticide effect of microorganisms seems to be the only and highly feasible way of thinking (Rodriguez and Fraga, 1999; Biró et al., 2000).

Tomato is one of the most frequent vegetable grown worldwide and consumed both in freshed and in processed forms (Helyes et al., 2014). This tasty vegetable has important role in healthy lifestyle, sustainable tomato production has therefore an increasing demand nowadays. In order to achieve the highest possible yield, unreasonable tillage and overuse of agrochemicals (pesticides, chemical fertilizers) are applied at intensive growing systems (Glendining et al., 2009). Such breeding practices on the other hand generally reduce the nutritional value of crops, vegetables and also of the fruits. Tomato on the other hand might contain significant amounts of natural health-protecting compounds, such as the organic acids, sugars and antioxidant materials, such as the lycopene, C-vitamins and others (Devi et al., 2008; Helyes et al., 2014).

Soluble sugars and organic acids play crucial role in tomato quality and food taste values. Tomato contains 5.0-7.5 % of dry matter that is mainly constituted of fructose, glucose, citric acid, malic acid, and other organic compounds (Sariyer and Oztokat, 2015; Salles et al., 2003). Major organic acids in tomatoes are citric- and malic acids, with higher dominance of citric-acids (Davies and Hobson, 1981). Levels of organic acids are highly depending on the ripeness and also on the certain cultivars, beside the crucial soil-environmental affecting factors (Baldwin et al., 1991; Béni et al., 2014, 2017). Phosphorus supply of tomato plants influences highly the quantity and quality of biologically active compounds in the fruits (Di Cesare et al., 2010). Several key-important compounds of organic acids and sugars were examined therefore by high performance liquid chromatography (HPLC) beside the total soluble solid (TSS) content from ripe fruits.

Effect of some commercially available microbial fertilizer (bioeffector) inoculations, including spore-forming bacteria was studied on the fruit quality parameters of tomato among controlled light-chamber and at field-conditions. Main focus was given for answering of the following questions: Is *Bacillus* spp. biofertilizer inoculation increasing the soil microbial counts and available P for plants? Is some of inside tomato fruit quality parameters improved by the bioeffector inoculations? Is there any positive correlation between the bioeffector treatments and fruit quality? Is results from pots can be predictable to up-scaled natural field condition?

Materials and methods

Experimental background

Experiments were carried out in pots and also among organic field conditions in 2014. There were 4 different treatments used in the pot experiment of applying 3 dm^3 of pots in 4 replicates (*Table 1*). Among field conditions there were 12 of 4×5m plots used at the Research and Experimental Farm of the Szent István University of Budapest, Horticultural Faculty, Ecological Farming Division in Soroksár, Hungary (N 47°40'; E

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19°15' at 111 m altitude). Ecological farming practices are carried out since more than ten years on the experimental field site. For nutrient supplementation we applied authorized substances used in organic farming: Viano (13% N; 660 kg ha^{-1}), Patentkali (30% K_2O; 10% MgO; 17% S; 1200 kg ha^{-1}). Regarding plant protection against soil borne plant pathogens during the vegetation periods, we used copper-sulphate-containing fungicide (Cuproxat FW-1) treatment according to the suggestion of suppliers. Experimental area was regularly watered of using drip irrigation system. Some of the soil characteristics and also some data about environmental climatic conditions of the area are shown in *Table 1*. In 2014 it was an excessively rainy growing season with almost 1.5-times higher precipitation than the usual average annual value.

Table 1. *Main characteristics of soil at Soroksár site, Hungary*

Soil parameters	Measured values
pH_{H2O}	7.9
C:N (A horizon)	9.92
NO_2+NO_3-N	8.5 mg kg^{-1} soil
P (CAL)	43 mg (100 g^{-1} soil)
K (CAL)	17 (100 g^{-1} soil)
Mg ($CaCl_2$)	10 (100 g^{-1} soil)
Soil type	slightly hummus sandy soil
Mean annual temperature	11°C
Mean annual precipitation	500 mm

Pot experiment was performed in the light-chamber of Department of Soil Science and Water Management of Corvinus University (now it belongs to Szent István University), Budapest, Hungary. Soil used in the pots, was originated from the field experimental station of Soroksár, Hungary. Light-period of pot experiment was 14 hours in a daily basis in accordance with the requirements of the tomato plants. Pots were placed in a controlled-temperature light room, programmed at a temperature ranging from 17 °C to 28 °C, while the light intensity (Lux) had been 25000 lx (Herrera et al., 2008). Characteristics of applied soil are shown in *Table 2*. Both in pots and in field experiments the tomato (*Solanum lycopersicon* Mill.) Hungarian cultivar of 'Mobil' were used. Seedlings were grown under controlled conditions and planted into their final place after the 14^{th} days, preferably at the same stage of maturity of each. The pot experiment was carried out between 31^{st} of March 2015 and 16^{th} of June 2015. In case of field trials, the seedling was grown up till the 25^{th} of March 2015 in cold plastic house, transplanting of small 2-leaves of seedlings was done to bigger pots at 9^{th} of May 2015. Final seedlings-transplantation to the field condition was performed between 20-26 of May 2015.

Regarding the plant parameters, a biweekly visual inspection of plant-parameters was done during the vegetation periods (data not shown), and a final biomass-production of fresh and dry weight of shoot was estimated. Fruits of tomato was regularly measured from each of the tomato plants, and a summary of the yield was calculated in each plots and in each treatment. In this study we are focusing on the soil- and fruit- quality parameters.

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Biofertilizer treatments and estimation of soil-characteristics

Commercially available bioeffectors (BE products), i. e. biofertilizers, were used for both at the pots and the field experiment. On the basis of the results achieved in pot experiment, combinations of BE with other product of biofertilizers were also tested among field conditions according to *Table 2*.

During general set-up of pot experiment, there were 2500 g air-dried soil put in each of the pots and one tomato seedling/pot was planted. Biofertilizers were applied on the basis of supplier suggestions. Bioeffector (BE) strains were used in the rates of 1.33 % (v/v), i.e. 0.2 ml for each plants Hungarian BR1 and BR2 products, as a 2-component-biological fertilizer was also applied according to the supplier's protocol, BR1: 0.1875 $ml.plant^{-1}$; BR2: 0.375 $ml.plant^{-1}$, shown in *Table 2*.

Ammonium lactate method (P-AL) was used to estimate available phosphorus content: 5g of soils were shaken in 100 ml of 0.1 M ammonium lactate and 0.4 M acetic acid (pH 3.75) for 1 hours and phosphorus content was estimated from the filtrate colorimetrically (Egner et al., 1960).

Table 2. *Treatments and density of bioeffector inoculums at the pot and field experiment*

Treatments	Bioeffector strains	Colony forming units in product ($CFU\ g^{-1}$)	Inoculation rates
C (Control)	No inoculums, only water	-	-
BE (Bioeffector)	*Bacillus amyloliquefaciens* FZB42	2.5×10^{10}	1.33 % (v/v), i.e. 0.2 ml/plant
BR1 (Biorex-1)	*Bacillus subtilis; B. thüringiensis; B. megaterium*	2×10^{10}	5L/ha, 0.1875 ml/plant
BR2 (Biorex-2)	*Azotobacter chroococcum: Azospirillum lipoferum; Pseudomonas putida.*	2×10^{10}	10L/ha, 0.375 ml/plants
BE+BR2	Combination of 2 products	$1.25\times10^{10}+1\times10^{10}$	0,2+0,375 ml/plants

Most probable number (MPN) method of sporeforming bacteria in soil capable of growing in nutrient broth (meat extract 3 g; peptone 5 g; pH 7±0.2) (Marshall, 1993; Downes and Ito, 2001) was used by a microplate method. 10-fold serial soil dilutions were made from 10^{-1} to 10^{-8} dilutions as described (Libisch et al., 2010). Average soil samples were collected at the 10^{th} and 14^{th} weeks of tomato growth, with the highest suggested microbial activity in soil-plant system (Biró et al., 2000). From each samples and from each dilution three parallel 20 µl aliquots were transferred to sterile polystyrene 96-well microplates, after 10 minutes stay in water bath at 80 ^{o}C to kill the vegetative cells. Microplates were incubated at 28°C for 1 week. The plates were tested after that for growth using the respiration indicator iodo-nitro-tetrazolium violet (INT) (Sigma-Aldrich) (Johnsen and Henriksen, 2009). 50µl INT solution (3g/L INT dissolved in water) was added to each well, and the plates were incubated overnight at 28°C. Metabolically active bacteria reduce INT to the corresponding formazan forming a purple precipitate. The number of growth-positive wells at each dilution was determined

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by visual inspection of the plates. The statistical method of Cochran was applied to calculate MPN values using the MPN calculator VB6 (Cochran, 1950). One-way Anova Tukey test with logMPN values and standard logMPN errors was used to determine whether MPN values of various pot experiment and field experiment soil samples were not significantly different at the 95 % confidence level (<0.05) (Mukherjee et al., 2006; Vladar et al., 2008).

Fruit quality assessments

Sugars are the major soluble solids in tomato fruit juice. Soluble solids concentration (Brix or TSS) can be determined in a small sample of fruit juice using a hand held refractometer. Total soluble solid content (TSS) of tomato fruits was determined by Atago® PAL-3 refractometer device (Cavalcanti et al., 2013). The fruits (skins and fruit-pulp) were homogenized with a blender and homogenates were stored at -25 °C until analysis. After hawing 2 g from each fruit puree samples were diluted in 2 ml deionized water, shake for 1 hour in dark and centrifuged for 10 min at 10000 rpm (Hettich Mikro 22R). One ml supernatant was then pipetted off and filtered through a 0.45-µm MILLEX®-HV Syringe Driven Filter Unit (SLHV 013 NL, PVDF Durapore), purchased from Millipore Co. (Bedford, MA, USA). WATERS High Performance Liquid Chromatograph (HPLC, Waters Co., 34 Maple Street, Milford, MA, USA) equipped with 2487 Dual λ Absorbance Detector (for determination of organic acids), 2414 Refractive Index Detector (for the determination of sugars) 1525 Binary HPLC Pump, In-Line Degasser, Column Thermostat (set at 40 °C) and 717 plus Autosampler controlled with EMPOWER™2 software was used for HPLC analysis. For separation of organic acids Shodex KC-811 column (8 mm × 300 mm) with Shodex RSpak KC-G guard column were used. Mobile phase was a 0.1 % aqueous solution of phosphoric acid. Flow rate was adjusted to 1 $ml.min^{-1}$, giving a pressure of 600±25 psi on the column at 40 °C. The volume injected on the column was 20 µl, the detection time was adjusted to 20 min. Detection was carried out at analytical wavelength of 220 nm. Retention times (in min.) of the standards were 7.19 for citric- and 9.10 for malic acids. For separation of sugars a Sugar-PakTM column was used placed in a thermostat at 90 °C. The mobile phase was a 0.0001 M aqueous solution of Ca-EDTA. Flow rate was 0.5 ml min^{-1}, resulting a pressure of 450±10 psi on the column. The 20 µl of injected volume was used, detection took 30 min. for each sample. Retention times of standards were 8.32 for sucrose, 10.93 for glucose and 11.77 for fructose. Two replicates were used. Concentrations were calculated from areas of corresponding peaks and expressed in $mg.100^{-1}$ g.

Statistical analysis

For evaluation of the results one-way ANOVA test was applied. Normality assumption was proven by Kolmogorov-Smirnov test (p>0.05; p=0.200) or Shapiro-Wilk test (p>0.05) and the homogeneity of variances was checked by Levene’s test (p>0.05). As the estimation was proven we applied Tukey HSD post hoc test. If it was not proven the Games-Howell post hoc test was applied. Pearson correlation analysis was used to estimate the interrelation among soil and tomato fruit quality parameters. The analysis is appropriate to know how the involved and studied variables are related to each other. Results are presented in *Figures* and in *Tables*.

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Results

Soil parameters with bioeffector application

MPN counts of sporeforming bacteria

Fig 1a is showing the most probable number (MPN) of cultivable microorganisms in the soils, treated or non-treated by bioeffectors in the pot experiments. According to the result of total plate count, normality assumption was proven by the Shapiro-Wilk test ($p>0.05$; $p_1= 0.715$; $p_2=0.883$) and the homogeneity of variances was checked by Levene's test. As the assumption was proven, [$F(7;32)=2.151$; $p1>0.05$; $p=0.066$] we applied Tukey HSD post hoc test. Differences among the treatments were not appeared significantly. However, it is apparent that the number (logMPN) of microorganisms in the BE-treated soils was enhanced tendentiously from April to July in the pot experiment (*Fig. 1a*).

Among the natural field conditions *Fig 1b* is showing the most probable number of microorganisms in the soil of the field experiments after 8 (July) and 14 (September) weeks of growth. According to total plate count results, normality was proven by Shapiro-Wilk test ($p>0.05$; $p1= 0.715$; $p2=0.883$) and homogeneity of variances was checked by Levene's test. The assumption was proven, therefore we used Tukey HSD post hoc test [$F_{July}(7;8)=0.015$; $F_{Sept}(7;19)=0.057$; $p>0.05$]. Differences among the treatments were not appeared significantly either any growing conditions. In case of pot experiment the number of microorganisms in BE-treated soils were increased but the same was not proven among the field conditions, at the studied periods. No differences among the various bioeffector treatments could be found at the sampled time (*Fig. 1b*).

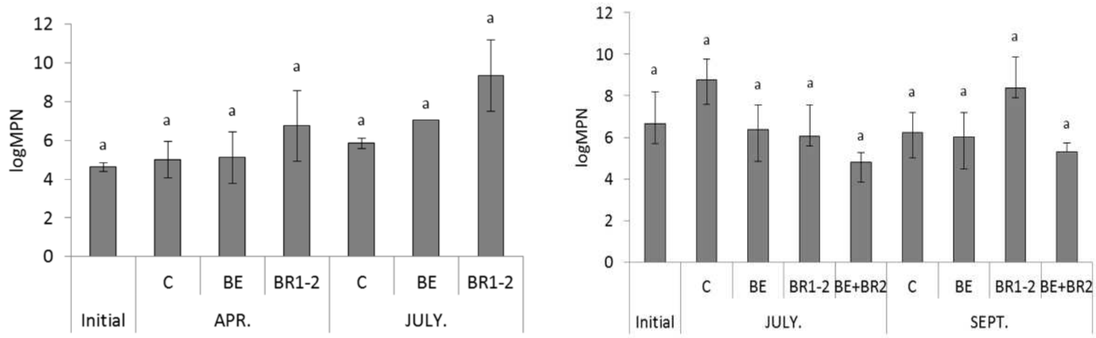

Figure 1a, b. *Most probable number of microorganisms (logMPN) in soils treated or not by various bioeffector products (**a**) and (**b**). Results in pots and in field, respectively (n=4)*

Soil-available phosphorus

Prior to the pot-trial a preliminary soil analysis was performed in soils, originating from the experimental field of Soroksár (Hungary), the site of the field tests on the same year. A specific focus was given for the availability of phosphor (P) doses, due to the fact, that used bioeffector products were predicted of containing mainly the specific P-mobilizing microorganisms. Among the macro elements, examined, phosphor content was found to be increased mainly in the pot experiment. Treatment with the presumably phosphorous-mobilizer *Bacillus* (BE, BR1) strains could increase the available P-content in the soil significantly (*Fig. 2a*).

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Among field conditions it was also examined that phosphorus mobilizer *Bacillus* (BE, BR1, BR2) strains could increase the available P-content in soils. Among treated and non-treated soils, however, no significant differences were recorded [F(3;8) = 2.269; p = 0.071] (*Fig. 2b*).

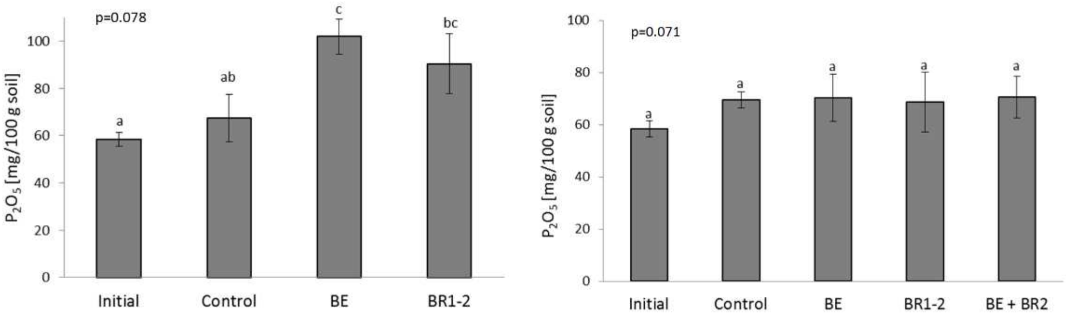

***Figure 2a, b.** Phosphorous, P_2O_5 content of soils after (**a**) 10- (in pots) and (**b**) 14 weeks (in field) growth of tomato (n=4)*

Fruit quality parameters with bioeffector application

Total soluble solids of tomato fruits

Tomato's fruit quality parameters were examined, by assessing the total soluble solid content (TSS). In case of pot experiment TSS value was significantly higher through the effect of used BE industrial product; while in case of BR1-2 treatments TSS value was significantly lower compared to the control. Post hoc comparisons using Games-Howell test indicated, that the mean score of BE (M=9.00; SD=0.608) and the BR1-2 treatment (M=5.2; SD=0.122) were significantly different from the control (M=6.62; SD=0.16) (*Fig. 3a*).

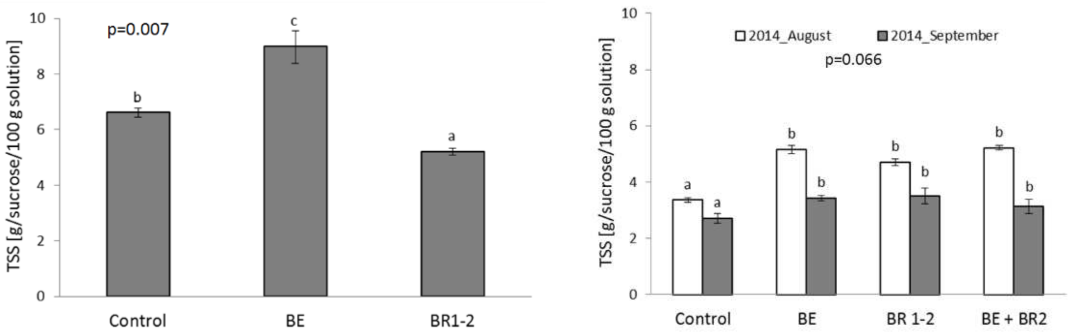

***Figure 3a, b.** Total soluble solid (TSS) content of tomato fruits after (**a**) 10 weeks of growth (in pots) and (**b**) as harvested after 10- (in August) and 14- (in September) weeks of growth (field experiment) (n=4)*

Among field condition beside the single BE treatment, combined effect of bioeffectors (BR1-2 and BE+BR2) was examined. In field the tomato fruits were harvested two times during the vegetation period (after 10 weeks, in August 2014, and after 14 weeks in September 2014). The fruit quality analysis was carried out on average samples after performing both harvests. In case of the field experiment TSS

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value of fruits from the treated plots were significantly higher than the control at both harvests. Post hoc comparisons using the Tukey HSD test indicated that the mean score of BE (M=5.16, SD=0.15), BR1-2 treatment (M=4.7; SD=0.02) and the BE3+BR2 combination (M=5.22; SD=0.08) were significantly different than the control (M=3.36; SD=0.089). In September the TSS value decreased but the differences have remained similar (*Fig. 3b*).

Sugar and organic acid content of tomato fruits

Among inside fruit quality parameters some of the sugar- (glucose, fructose) and the organic acids (citric-, malic-) components were assessed by high performance liquid chromatography (HPLC) both in pots and in field experiments. In comparison with total soluble solid (TSS) values, similar result appeared with the HPLC-measured sugar components, although those results were found to be lower. Regarding the sugar content in the pot experiment there were no significant differences found in comparison with the control but differences were significantly supported between the two treated groups. TSS value was found to correlate slightly with the concentration of sugar component measured by HPLC. Normality assumption was proven by Kolmogorov-Smirnov test ($p>0.05$; $p1= 0.200$; $p2=0.117$) and the homogeneity of variances was estimated by Levene's test As the assumption was not proven [$F1=F2(3;4)=0.005$; $p<0.05$] a Games-Howell post hoc test was applied (*Fig. 4a*).

The organic acids (citric- and malic) were also assessed by HPLC analysis. Regarding the pot experiment the organic acid content was significantly higher in case of control (M=565.79; SD=3.46) than the BE (M=525.39; SD=2.79) and BR1-2 treatments (M=261.03; SD=3.25) (*Fig. 4b*).

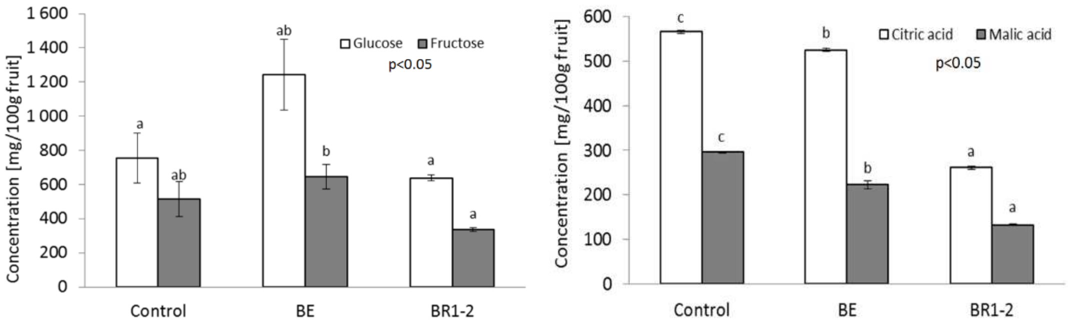

Figure 4a, b. *Sugar (glucose and fructose) (**a**) and organic acid (citric-, malic-) (**b**) content of tomato fruits after 10 weeks of growth in pot experiment (n=4)*

In case of the sugar content in tomato fruits from the field experiment, no significant differences were found in fructose and in glucose contents when single BE treatment was used. When two harvesting periods were compared, the same results were achieved, so no significant differences were found in the sugar concentration. Both normality assumption was proven by Kolmogorov-Smirnov test ($p>0.05$; $p_{Aug}= 0.200$; $p_{Sept}=0.117$) and the homogeneity of variances was estimated by Levene's test. As the assumption was not proven [$F_{Aug}=F_{Sept}(3;4)=0.005$; $p<0.05$] a Games-Howell post hoc test was applied. Post hoc comparisons using the Games-Howell test indicated that the mean score of BE, BR1-2 treatments, and the BE+BR2 combination were not significantly different than the control (*Fig. 4a*).

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The organic acids (citric- and malic) were also assessed by HPLC analysis. In the field experiment two sampling periods and measurement of the organic acids were applied. In September there was an increasing amount of citric acid in tomato fruits, grown in the bioeffector treated soil. The same only tendentiously was supported for malic acid content (*Fig. 5b*).

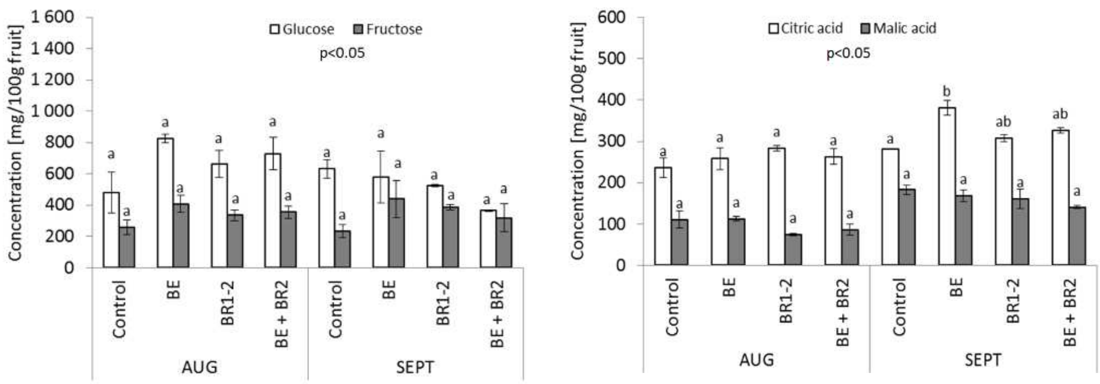

Figure 5a, b. *Sugar (glucose and fructose) (**a**) and organic acid (citric-, malic-) (**b**) concentration of tomato fruits after 10 (August) or 14 (September) weeks of growth in field experiment (n=4)*

Correlation among soil characteristics and tomato fruit quality parameters

Correlation analysis was performed among total cultivable sporeforming microorganisms (*Bacillus* sp.), estimated by MPN (Most Probable Number) method and some of the tomato fruit quality parameters both at pot- and at field experiments.

Correlation results of MPN, measured TSS values and estimated inside fruit quality parameters are shown in *Table 3* and *Table 4*. Interrelation of inside fruit quality with the used biofertilizer application was found to be greater in pot experiment, in comparison with the field experiment with more variable environmental condition.

Results of pot and field experiments are summarized in *Appendix 1*. Measured data of microbial soil and fruit quality parameters are detailed, as the average of 4 replicates, with the standard errors.

Table 3. *Correlation among soil microbial abundance of sporeforming bacteria (MPN), the total soluble sugars (TSS) and inside fruit quality parameters, soluble sugars (glucose, fructose) and organic acids (citric-, malic-) in tomato fruits, grown in pot experiment (n=4)*

Correlation values (pot experiment)						
	MPN	**TSS**	**Glucose**	**Fructose**	**Citric-acid**	**Malic-acid**
MPN	1	**0,788****	0,666	0,643	0,473	0,261
TSS	**0,788****	1	**0,945****	**0,936****	**0,708***	0,432
Glucose	0,666	**0,945****	1	**0,910****	0,520	0,213
Fructose	0,643	**0,936****	**0,910****	1	**0,804****	0,582
Citric-acid	0,473	**0,708***	0,520	**0,804****	1	**0,941****
Malic-acid	0,261	0,432	0,213	0,582	**0,941****	1

**. Correlation is significant at the 0.01 level; *. Correlation is significant at the 0.05 level.

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Table 4. *Correlation among soil microbial abundance of sporeforming bacteria (MPN), the total soluble sugars (TSS) and inside fruit quality parameters, soluble sugars (glucose, fructose) and organic acids (citric-, maleic-) in tomato fruits, grown among field condition (n=4)*

Correlation values (field experiment)						
	MPN	TSS	Glucose	Fructose	Citric-acid	Malic-acid
MPN	1	0,192	-0,459	-0,490	0,423	-0,463
TSS	0,192	1	0,015	-0,160	**0,880****	**-0,586***
Glucose	-0,459	0,015	1	**0,965****	0,276	0,297
Fructose	-0,490	-0,160	**0,965****	1	0,113	0,431
Citrit-acid	0,423	**0,880****	0,276	0,113	1	-0,222
Malic-acid	-0,463	**-0,586***	0,297	0,431	-0,222	1

**Correlation is significant at the 0.01 level; *. Correlation is significant at the 0.05 level.

Discussion

"Biofector" is an integrated international project with the aim to reduce input of mineral fertilizers in European agriculture by development of specifically adapted bio-effector microorganisms (BEs) to improve the efficiency of alternative fertilization strategies, such as organic and low-input farming, use of fertilizers based on waste recycling products and fertilizer placement technologies. Bioeffectors addressed comprise fungal and bacterial strains (e.g. *Bacillus*, *Trichoderma*, *Pseudomonas* etc.) with well-characterized root growth promoting and nutrient solubilizing potential. In Biofector international project one of the test plants is tomato, which is important in many ways in human consumption. Significant industrial and commercial use is known, exceeding all of the vegetables in terms of both cultivation and consumption in World. The aim of Biofector research project is the development of viable alternatives to conventional mineral fertilization, and contribution to a more efficient management of non-renewable resources of mineral nutrients, energy and water, to preserve soil fertility and to counteract the adverse environmental impact of agricultural production. Our study is part of the above mentioned research.

Tomato is very sensitive to the P supply. It is required relatively high amount of phosphorus, especially at the beginning of its growth (Schmidt et al., 2010). For this reason, some of the marketed bioeffector products were used in this study, which are known to solubilize and mobilize the highly available phosphorus content in the soil. The general use of *Bacillus* spp. strains as P-mobilizing microorganisms are also supported by the fact that those microbes can survive in the soils among several, even at the most serious environmental stress conditions. Due to its spore forming ability the cells of the *Bacillus* spp. can survive long in the soils and at any potential environmental conditions. It is why the individual species and *Bacillus* spp. genus are considered as one of the microbial groups, with specific food-quality and safety importance (Beczner et al., 2004; Dudás et al., 2014; Kocsis and Biró, 2015). Among the marketed products, which are containing spore forming bacteria there were single and combined inoculation treatments used in this study. It was hypothesized that the more types of bacteria, including more than one particular species could produce better performance for the plant growth promotion ability. This idea was already supported by several authors of using different combinations of bacteria or fungal-bacterial consortium, with an uppermost importance of such systems in polluted or environmentally stressed

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ecosystems (Kloepper et al., 1989; Biró et al., 1993; Mantelin and Touraine, 2004; Vivas et al., 2006). Among sustainable agricultural practice the synergism of symbiont type of microbes is also demonstrated between arbuscular mycorrhiza fungi and the nitrogen-fixing bacteria. The behaviour of microbes in a consortium is dependent on several environmental factors. It has been reported, that even a beneficial symbiosis is diminished in a short time of periods when the environmental condition, such as the drought, high temperature or reduction of light intensity or heavy metals toxicity become non-tolerable to the macro- and micro-symbionts (the higher plants and the microbes) (Biró et al., 2000, 2015; Füzy et al., 2014). In case of the microbes are tolerable to severe environmental stress-condition, the symbiosis with a joint strategy might be helpful, finally resulting the better survival and growth of host-plants.

The abundance of inoculated microorganisms was followed by the most probable number (MPN) method. The quantity of cultivable spore-forming microbes were estimated, by preliminary kill of the non-spore-bearing vegetative cells from the soil-suspension. Although the presence or absence of those inoculated microorganisms was assessed only occasionally at the beginning and at 2-times during the vegetation periods, there were not greater abundance recorded as the total countable number in the tomato rhizosphere. This result indicates the potential of fast microbial changes in the rhizosphere and the interrelation among microbial colonization with plant growth parameters. Similar conclusion was made by Biró et al. (2015) who found that this effects can be highly dependent on the soil quality, the nutrient content of soils, more particularly focusing on the soil available nutrients during plant growth. On the basis of Kocsis et al. (2015) this type of slightly hummus sandy soil are low in available nutrients and in specific microbes. Significantly increase the microbial abundance is necessary to use soil improvers (e.g. biochar) or organic fertilizer, compost application, which might enhance soil microbial status in one step. We found only in the pot experiment that the abundance of microorganisms is increasing with the plant age. The importance of microbial inoculation is highlighted at early seedling status, when the PGR hormone-effect of inoculated microbes might enhance root-volume, indirectly improve water- and nutrient-uptake of plants (Yang et al., 2009; Biró et al., 2000; Choudry et al., 2015). The connection of microbial presence and activity with plant-necessity was already demonstrated among serious environmental stress conditions in several previous publications (Vivas et al., 2006; Domonkos et al., 2010; Panwar et al., 2011).

Testing the MPN abundance of specific sporeforming microorganisms in the tomato plant rhizosphere, differences among the used treatments were not found significantly in our study. It was apparent, however that the number (logMPN) of studied microbes in the BE treated soils was enhanced tendentiously with plant age (from April to July) in the pot experiment. It was also demonstrated in this study, that the abundance was found parallel with the available phosphorus concentrations in the soil. In connection with this finding Fekete et al. (2011) found, that more organic P can enhance the alkaline phosphatase activities, which finally increase the P-availability in the soil. The alkaline phosphatase is mainly produced by the soil microorganisms and the extent of its synthesis and excretion of it can be coupled to the microbial activity and/or the population size, as it was shown by Kaleeswari (2007) and Garg and Bahl (2008). Due to the presence of BE *Bacillus amyloliquefaciens* FZB42 an increased phosphorus content was found in the treated pots.

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Regarding the size and number of fruits there was also an increase in treatment BE and BR1-2 in the pot experiment (data not shown). Richardson et al. (2001) and Idriss et al. (2002) made a similar finding in their experiments in laboratory and field conditions with the same *Bacillus amyloliquefaciens* species. They found that improved phosphorus nutrition is achievable by mobilization of phosphorus fixed as insoluble inorganic polyphosphates which accounts for 20-50 % of the total soil organic phosphorus. It is generally accepted that higher microbial counts can result higher nutrient availability in soil. Contrarily, in case of the combined treatments, where beside the *Bacillus* spp. strains some other microorganisms, as the Nitrogen-fixing *Azospirillum* and *Azotobacter* furthermore the siderophore-producing *Pseudomonas* bacteria (BE+BR-2) were given, no further improved growth parameters were recorded on tomato.

Considering the fruit quality there are also several study of showing the importance of biofertilizer application. According to Bruulsema et al. (2004), phosphorous fertilization is a key component in the metabolism and regulation of several pathways involved in the biosynthesis of secondary plant metabolites. Many of those materials are biologically active compounds. P may increase the level of some acids such as ascorbic acid, although interaction with climatic factors and the growing season growing area may occur. The high level of citric acid concentration was found at a high level of phosphorus concentration by Di Cesare et al. (2010). Tóth et al. (2007), Oke et al. (2005) studied the effect of P fertilizer on the quality of tomato under field conditions for three consecutive years, by evaluating the pH, the acidity, the lycopene, the vitamin C content and also the flavour volatiles. They noted that the influence of P application on several of the quality parameters mentioned above was marginal, while climatic conditions had a more predominant effect. Organic acids and sugar comprise the majority of the total dry matter content of tomatoes (Malundo et al., 1999). The “reducing type” of sugars and organic acids are significant components of fruits, determining the sweet- and the sour taste of the tomatoes, respectively. Their concentration may also affect flavours acceptability (Salles et al., 2003). Tomato fruits harvested at the same time from the bioeffector-treated plots had a more balanced acid-sugar ratio and the control had higher level of organic acid concentration. The well-balanced acid-sugar ratio appreciably increases the food quality and tasty value of the tomato fruits.

On the bases of this study we found that phosphorus mobilizer microorganisms can be successfully used as bio-fertilizers in ecological farming systems, since through applying them soluble phosphorus of the soil might be increased under certain environmental conditions. Phosphorus content of the soil was higher as a result of the used bioeffector inoculation, presumably capable for phosphorus mobilization. As a consequence, the nutritional and food quality and tasty value of tomato fruits (the acid-TSS ratio) have changed to a more favourable, tasteful direction. A tastier, more marketable food was produced by an ecological organic way. Governed by the principles of sustainable farming methods, the application of artificial agrochemicals can be reduced, including also the inorganic phosphorus fertilizer application. Eco-friendly, natural bio-fertilizers and combined bioeffective solutions might play important role in the sustainable agri-/horticultural practices.

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APPENDIX

Appendix 1. *Summarised data of estimated soil characteristics and tomato fruit quality parameters in the pot and field experiments with bioeffector inoculations (Budapest, Soroksár, 2014) (n=4)*

Data of pot experiment (average+/-standard error)								
Treatments	Harvest (weeks)	MPN*	Phosphorus (mg/100g soil)	TSS (g/sucrose/100g solution)	Glucose (mg/100g fruit)	Fructose (mg/100g fruit)	Citric acid (mg/100g fruit)	Malic acid (mg/100g fruit)
Control	10	5,42+/-0,61	67,4+/-19,1	6,62+/-0,16	754,5+/-147,4	516,3+/-102,6	565,8+/-3,5	295,4+/-1,3
BE	10	6,07+/-0,66	101,9+/-7,3	9,08+/-0,74	1244,3+/-206,3	645,9+/-71,3	525,4+/-2,8	222,2+/-9,9
BR1-2	10	8,05+/-1,83	90,4+/-12,7	5,20+/-0,12	638,6+/-16,6	337,3+/-0,4	261,0+/-3,2	132,2+/-1,9
Data of field experiment (average+/-standard error)								
Treatments	Harvest (weeks)	MPN*	Phosphorus (mg/100g soil)	TSS (g/sucrose/100g solution)	Glucose (mg/100g fruit)	Fructose (mg/100g fruit)	Citric acid (mg/100g fruit)	Malic acid (mg/100g fruit)
Control	10	6,80+/-0,72	69,5+/-3,0	3,36+/-0,05	480,6+/-131,0	258,3+/-45,3	236,7+/-23,2	110,9+/-21,3
	14			2,70+/-0,17	631,6+/-58,4	231,8+/-41,7	281,2+/-0,1	182,7+/-10,7
BE	10	6,30+/-1,08	70,2+/-9,1	5,16+/-0,15	827,3+/-164,6	408,6+/-56,1	258,0+/-26,6	112,8+/-4,9
	14			3,42+/-0,08	579,7+/-164,6	439,3+/-119,8	380,8+/-17,8	168,1+/-13,7
BR1-2	10	6,02+/-1,35	68,7+/-11,5	4,70+/-0,01	662,4+/-86,6	355,2+/-56,1	262,9+/-18,4	74,7+/-2,4
	14			3,50+/-0,28	523,5+/-8,5	384,3+/-17,4	307,6+/-7,6	161,4+/-23,5
BE+BR2	10	6,59+/-0,99	70,5+/-68,7	5,22+/-0,08	728,1+/-103,9	355,2+/-38,9	262,9+/-18,4	85,8+/-13,2
	14			3,14+/-0,25	364,5+/-2,8	319,6+/-86,8	326,2+/-6,0	140,9+/-4,4

*Most probable number count of sporeforming bacteria (log10) in soil.
Treatments: BE - Bioeffector treatment, inoculated by *Bacillus amyloliquefaciens* FZB42 strain (RhizoVital); BR1 - mixture of *Bacillus subtilis, B. thüringiensis, B. megaterium* strains (Biorex product); BR2 - mixture of *Azotobacter chroococcum, Azospirillum lipoferum* and *Pseudomonas putida* strains (Biorex product)

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ESTIMATION OF NICOTINE CONTENT IN TOBACCO LEAVES BASED ON HYPERSPECTRAL IMAGING

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Abstract. We used spectroradiometry to rapidly and nondestructively predict the nicotine content in flue-cured tobacco leaves and obtained hyperspectral images of tobacco leaves during the squaring development stage at field conditions. We tested the nicotine content in the lab and analyzed correlations between 28 hyperspectral characteristic parameters and the nicotine content of tobacco leaves to establish inversion models. The result showed that (SDr−Sy)/(SDr+Sy), Rg/ Ro, and (Rg−Ro)/(Rg+Ro) provided good correlations for the nicotine content; an inversion model based on (SDr−Sy)/(SDr+Sy) in relation to the nicotine content, $Y=-4.2628+5.8974X_{(SDr-Sy)/(SDr+Sy)} -1.3260X_{(SDr-Sy)/(SDr+Sy)}2$, and a multiple regression model based on Rg/Ro, (SDr− Sy)/(SDr+Sy) in relation to the nicotine content, $Y=-0.4753+1.6982\ X_{(SDr-Sy)/(SDr+Sy)} -0.1616X_{Rg/Ro}$, could be used to predict the nicotine content of flue-cured tobacco leaves during the squaring stage.
Keywords: *flue-cured tobacco, spectral reflectance, chemical composition, inversion model, prediction*

Introduction

Tobacco was used in smoking products and nicotine was the most important chemical that influences the quality of tobacco, and it causes aphysiological dependence (Xu et al., 2003; Li et al., 2007). The nicotine content in tobacco leaves was an important indicator for the quality of the raw tobacco material; thus, it must be monitored to maintain a consistent and stable cigarette quality (Yan et al., 2001; Weeks et al., 1995).

It was a timely, convenient and nondestructive monitoring method to use hyperspectral reflectance to predict nicotine content in tobacco leaves (Xing et al., 2016). Near-infrared analysis was currently the most convenient way to detect the nicotine content after baking (Qiao, 2015). However, there was no monitoring method that can be applied to large tobacco fields. Many studies have examined the biochemical components and nutrient elements based on reflection spectral characteristics (Tang et al., 2003; Tong et al., 1997; Shibayama and Akiyama, 1989, 1991) and accordingly hyperspectral inversion prediction model was established. The results show that the optimum predicted method for nicotine content in tobacco leaves was the linear fitting of the two order differential of 1135nm reflectance (Li et al., 2006a). It was feasible to

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predict the main chemical composition of a crop using hyperspectral images (Su et al., 2013; Zhao et al., 2014). However, most hyperspectral data was obtained in a controlled environment (Fang et al., 2013; Wang et al., 2011; Zhang and Zhao, 2000) hence, the inversion model and application effect have some limitations. So, there was still no effective and timely monitoring means for large areas of tobacco leaves. We selected a relatively concentrated producing area to examine the relationship between the hyperspectral parameter and the nicotine content in flue-cured tobacco under natural conditions. We set up an inversion model to predict the nicotine content in flue-cured tobacco leaves. We believe that the experiments will provide a good foundation for predicting nicotine content of large-forecast tobacco.

Materials and Methods

Study area

The study was carried out in a relatively concentrated flue-cured tobacco production area of Lin-yi, China. The study area was located in in the northern part of Yishui county, Shandong Province (Latitude35°36′～36°13, longitude 118°13′～119°03′). The topography of study area was relatively unitary, and tobacco planting was carried out by the unified organization of the tobacco companies, unified management, unified planting, unified fertilization. Flue-cured tobacco varieties was NC102.

Sampling design

A total of 100 spatially isolated tobacco plants were sampled to determine their leaf-level nicotine content, of which 70 samples were used for modeling, and 30 samples were used. We identified individual tobacco sample locations by Gridding method. Depended on the green depth of tobacco leaves, locating these points in the field, and then selecting the nearest “suitable” tobacco plant. For the purpose of this study, thesanples divided into three groups based on the color depth, which represents nitrogen under nutrition, normal nutrition,or nitrogen over nutrition. All data were recorded at midday (10:00–14:30, GMT+8) under clear skies and windless conditions.

Live tobacco measurements

Green-leaf samples were collected from each tobacco plant. Reflectance spectra (350～2500 nm) were collected for optically dense ground leaf samples using a high-intensity contact probe attachment (Analytical Spectral Devices (ASD) Inc., Boulder, CO, USA). The spectral resolutions of the instrument are 3 and 10 nm and the sampling intervals are 1.4 and 3.0 nm in the 350～1000 and 1000～2500 nm regions, respectively. The samples were killed at 105 °C for 15 min and then oven dried (60 °C for 72 h), passed through a 1-mm mesh screen, and weighed to the nearest 0.1 mg. The leaf-level nicotine concentrations (grams of N/100 g sample) of the oven dried ground foliage were determined using a LECO TruSpec CN analyzer (St. Joseph, MI, USA).For each sample, a series of twenty replicates were obtained, and the probe was then re-calibrated using a white reflectance cap accessory available from the manufacturer.

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Spectral data processing

Leaf-level reflectance data were transformed using standard derivative analysis and continuum removal methodologies (Xiao, 1998). Both transformations were applied directly to the spectrometer data and/or to a smoothed version of the spectrometer data. We opted to smooth the spectrometer data by resampling using coarser HyMap sensor channel configurations (472～2488 nm; 15～20 μm bandpass and sampling interval) because we are familiar with the sensor and the HyMap imagery has been successfully used for remote sensing of foliar biochemistry. Standard derivative analysis was implemented by calculating first derivative reflectance (FDR) values for log-transformed reflectance data (log10 (1/R), where R was reflectance) using the ViewSpec Pro software (version 6.0, ASD).The hyperspectral indices were chosen according to previous studies(Huang et al., 2014; Liang et al., 2013; Li et al., 2006b; Deng et al., 2008; Wang et al., 2011; Xia et al., 2009; Hu et al., 2001; Breuer and Pavan, 1955; Nevalainen et al., 2014; Li et al., 2013; Yang et al., 2011) and are presented in *Table 1*. The related coefficients between the nicotine content and vegetation index were calculated using Data Processing System (DPS) software (Canfield Clinical Systems, Fairfield, New Jersey).A model fitting effect was tested using the relative error (RE), determination coefficient (R^2), and root-mean-square error (RMSE).

Results

Correlation analysis for tobacco leaf nicotine content and high spectral index

A significant correlation was observed between the six indexes of the tobacco leaves and the nicotine content (*Table 1*). The correlation coefficients for five hyperspectral indices and the nicotine content of tobacco leaves were extremely significant. The absolute value of the correlation coefficient between the (SDr−Sy)/(SDr+Sy) index and the tobacco leaf nicotine content was above 0.80. The absolute values of Rg/Ro, (Rg−Ro)/(Rg+Ro), and the nicotine content of the tobacco leaves were all above 0.40.

Table 1.*The correlation coefficients between the nicotine content of tobacco leaves and the spectral parameters (n=70)*

Index	Correlation Coefficient	Index	Correlation Coefficient
Db	-0.1677	SDr/ Sy	-0.1930
λb	0.2273[a]	SDr/ SDg	0.1621
SDb	-0.2481[a]	(Rg−Ro)/(Rg+Ro)	-0.4014[b]
Dy	0.2550[a]	(SDr−SDb)/(SDr+SDb)	0.3080[a]
λy	-0.010	(SDr−Sy)/(SDr+Sy)	0.8707[b]
Sy	0.1838	(SDr−SDg)/(SDr+SDg)	0.1650
Rg	-0.1371	NDSI(1350, 700)	0.2043[a]
SDg	-0.1316	NDSI(FD700, FD690)	0.3496[b]
Ro	0.0978	NDVI(573, 440)	-0.3980[b]
Rg/ Ro	-0.4152[b]	NDVI(660, 440)	-0.2986[a]
SDr/ SDb	0.2804	NDVI(1220, 610)	0.2081[a]

[a]$P \leq 0.05$, [b]$P \leq 0.01$

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Establishment and testing of the nicotine content estimation model for tobacco leaves

The parameters for the correlation coefficient of the nicotine content in different growth stages for the tobacco leaves (independent variables) were extremely significant. A linear function ($y=a+bx$), power function ($y=ax^b$), logarithmic function ($y=a+b\ln(x)$), quadratic function ($y=a+bx+cx^2$), exponential function ($y=ae^{bx}$), and negative exponential function ($y=a/(1+\exp(b+cx))$)were chosen as mold functions to establish a tobacco leaf nicotine content inversion model (where y represents the nicotine content of the tobacco leaves and x represents the hyperspectral eigen values; a, b, and c are constants). Then, the Root Mean Square Error (RMSE) and Relative Error (RE) were selected to evaluate the fitting effect between the estimated value and the measured value to verify the accuracy and applicability of the model. Two hyperspectral indices, Rg/Ro and (SDr−Sy)/(SDr+Sy), with high correlation coefficients were chosen as the independent variables for the estimation model to establish the univariate estimation model and multiple regression estimation model.

Establishment and testing of the univariate estimation model for nicotine content in tobacco leaves

The inversion model was established using the hyperspectral indices, Rg/Ro and (SDr−Sy)/(SDr+Sy), as independent variables and nicotine content as the dependent variable (*Table 2*).

Table 2. *Nicotine concentration retrieval model for tobacco leaves during the squaring development stage and the test results*

Index	Estimation model	R^2 (n=70)
(SDr−Sy)/(SDr+Sy)	$y = -1.1977+1.7989x$	0.7582^{**}
	$y = 2.2427/(1+\exp(6.1463-4.7476x))$	0.7955^{**}
	$y = 0.7597x^{1.6421}$	0.7210^{**}
	$y = 0.3258\times\exp(0.9926x)$	0.6667^{**}
	$y = -4.2628+5.8974x-1.3260x^2$	0.8112^{**}
Rg/Ro	$y = 3.0047-0.4399x$	0.1724
	$y = 2.1130/(1+\exp(-4.5275+1.0456x))$	0.1784
	$y = 4.7454x^{-0.9553}$	0.1557
	$y = 4.0603\times\exp(-0.2945x)$	0.1669
	$y = 2.3793-0.0833x-0.0498x^2$	0.1734

From the data in *Table 2*, the decisive coefficients of the univariate inversion model (Eq. 1) and (Eq. 2) with the hyperspectral parameter (SDr−Sy)/(SDr+Sy) as the independent variable were all significant.

$$Y = 2.2427/(1+\exp(6.1463-4.7476X_{(SDr-Sy)/(SDr+Sy)})) \quad \text{(Eq. 1)}$$

$$Y = -4.2628 + 5.8974X_{(SDr-Sy)/(SDr+Sy)} - 1.3260X_{(SDr-Sy)/(SDr+Sy)^2} \quad \text{(Eq. 2)}$$

The estimation model was tested using the nicotine content data from 30 tobacco leaf samples, and the estimated RMSE and RE (%) were used to evaluate the fitting effect of

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the model. The R^2 value was high and the RMSE and RE (%) values were small for the inversion model (Eq. 1) and (Eq. 2).

Establishment and test of the multiple regression model for nicotine content in tobacco leaves

The multiple regression model was established using Rg/Ro and (SDr−Sy)/(SDr+Sy) models (with higher correlation coefficients) as the independent variables. The regression equation was (Eq. 3).

$$Y = (-0.4753 + 1.6982X_{(SDr-Sy)/(SDr+Sy)} - 0.1616X_{Rg/Ro}) \quad \text{(Eq. 3)}$$

F = 135.7792, p = 0.0000, R = 0.8827, and R^2= 0.7791 (adjusted correlation coefficient, Ra = 0.8794; adjusted coefficient of determination, Ra^2 = 0.7734). The nicotine content of the tobacco leaves was predicted using the model (Eq. 3) and the correlation coefficient between the predicted value and the measured value was 0.8841; RMSE =0.2335 for the prediction equation and RE = 15.47%.

Optimum estimation model of nicotine content in tobacco leaves

Single-variable prediction model

Based on the above analysis, the nicotine content of the tobacco leaves from the single-variable prediction model was Eq.2.

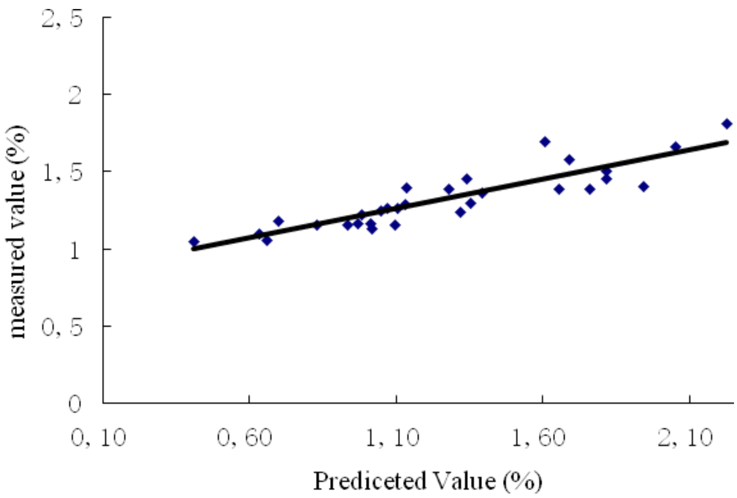

Figure 1. *Validation result of estimation model of nicotine content of tobacco*

The estimated RMSE and RE (%) were used to evaluate the fitting effect of the model. The correlation coefficient of the fitted equation was 0.8148. The RMSE of the fitted equation was 0.2272 and the RE was 14.42%. Further tests for the accuracy of the model (Eq. 2), provided the measured nicotine value and the estimated value of the 1:1 diagram. The correlation coefficient between the predicted value and the measured value was 0.7894. The model provides good precision and can be used to predict the nicotine content in tobacco leaves.

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Multivariate prediction model

Based on the above analysis, the nicotine content of tobacco leaves from the multivariate prediction model was Eq.3.

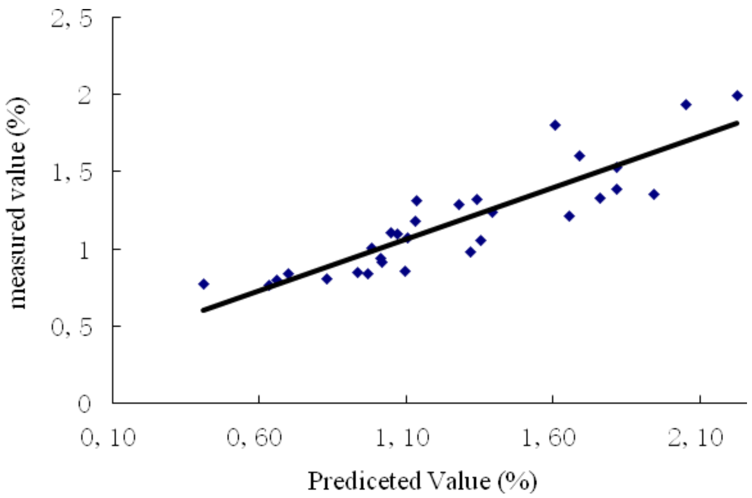

Figure 2. *Validation result of estimation model of nicotine content of tobacco*

The estimated RMSE and RE (%) were used to evaluate the fitting effect of the model. The correlation coefficient of the fitted equation was 0.8841. The RMSE of the fitted equation was 0.2335 and the RE was 15.47%. Further tests for the accuracy of the model (Eq. 3), provided the measured nicotine value and the estimated value of the 1:1 diagram. The correlation coefficient between the predicted value and the measured value was 0.7816. The model provides good precision and can be used to predict the nicotine content in tobacco leaves.

Discussion

Although there are technical standards and uniform requirements for tobacco cultivation in China, annual and regional differences of the nicotine content are significant because of the effects of natural precipitation, soil fertility, and field management measures. Nicotine was the most important tobacco leaf quality factor, and its content determines the intrinsic quality of tobacco leaves and the status of the formula. Industrial enterprises need to predict the main chemical components (especially nicotine content) of tobacco leaves before tobacco procurement, and reduce annual chemical composition differences of tobacco leaves by allocating different tobacco production plans to maintain a stable quality for tobacco brands. This study could only predict the nicotine content of fresh tobacco leaves by screening hyperspectral parameters, establishing models, and optimizing the validation methods. However, we did not establish the relationship between the spectral characteristic parameters and the nicotine content of the initial flue-cured tobacco after preparation. The method could not provide a practical basis for the changes of the cigarette tobacco leaf and recipe directly from the operational level. Future work would include exploring

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the precise labeling of hyperspectral tobacco leaves, studying the nicotine content of the first flue-cured tobacco, establishing an estimation model, and predicting the nicotine content of the early flue-cured tobacco to provide the basis for tobacco leaf dispensing and formulation allocation to the tobacco industry.

Conclusion

Using tobacco plants with different nicotine contents growing in natural field conditions, we analysed its hyperspectral characteristics, the Correlation between hyperspectral parameters and nicotine content. Hyperspectral parameters related to nicotine content in tobacco leaves have been preliminarily established and A prediction model of nicotine content of tobacco leaves with high precision was established. The results show that there was a high correlation between the tobacco nicotine content and the (SDr− Sy)/(SDr+Sy), Rg/Ro, and (Rg− Ro)/(Rg+Ro) parameters. The equations for the univariate estimation models (1) and (2), can be used to predict the nicotine content of flue-cured tobacco leaves. The multivariate regressive estimation model equation (3) was established using hyperspectral parameters Rg/Ro and (SDr−Sy)/(SDr+Sy) and can be used to predict the nicotine content in flue-cured tobacco leaves. The results of this study and the estimation model are closer to the actual situation for the flue-cured tobacco growth and development environment, and can provide a reference for the prediction of tobacco nicotine content in large areas.

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MEAL AND OIL QUALITY AMONG GENOTYPES OF INDIAN MUSTARD (*BRASSICA JUNCEA*) VARIES UNDER RECOMMENDED DOSE OF NITROGEN FERTILIZER

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Abstract. Out of the 24 genotypes of Indian mustard 63 % showed increase in total seed nitrogen content after 80 kg N (N80) application while 37 % showed a decrease compared to control (N0). In some genotypes the increase in total seed nitrogen content under N80 had no influence on total soluble protein. About 88 % genotypes showed reduction in crude fiber by 1.10 % (GM-2) to 35.6 % (DRMR-IJ-31) and in all 24 genotypes soluble sugars also reduced by 8% (Maya) to 80% (HB-9912) suggesting the influence of N application on digestibility of meal. Profiling of seed storage proteins showed differences in banding patterns in treated samples with prominent changes in α and β chains of cruciferin. In case of SFA and ω6:ω3 ratio, 50% of genotypes showed increase while 50 % genotypes showed a decrease in their SFA and ω6:ω3 ratio compared to control. Oil stability index also increased in 63 % of the genotypes under N80. Undesirable Erucic acid was found to increase in 58 % of the genotypes by 0.98% (DHR-991) to 38.31% (HB-9902) and in rest of the 42 % it reduced by 1.14 % (DRMR-IJ31) to 22.55 % (NRCDR-2) over control (N0).
Keywords: *total phenol, ascorbic acid, total soluble sugars, crude fiber, seed storage protein*

Introduction

Indian mustard is a winter oilseed crop largely grown in semi-arid regions of northern India. It is considered healthy and ideal for cooking because of low saturated fatty acid (SFA), high monounsaturated fatty acid (MUFA), moderate polyunsaturated fatty acid (PUFA) content, ideal ω6/ω3 ratio, low acid value, low saponification value and high smoke point. Besides high quality oil, the seed meal has high protein content which is ideal for animal feed or even fit for human consumption (Wanasundara, 2011). The high protein content also implies a high absorption of nitrogen from the soil which makes nitrogen fertilizers very important for this crop.

Plants take up nitrogen in the form of nitrate, as well as ammonium, this in turn can modulate the uptake of other anions and cations causing a change in the primary and secondary metabolic process and consequently the quality of oil and meal. Optimization of nitrogen application is important for the synthesis of C-based phytochemicals, including natural antioxidants such as phenolic groups (Björkman et al., 2011). Some

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reports have shown decrease in phenol content under higher doses of nitrogen fertilizers (Li et al., 2008; Giorgi et al., 2009; Zaghdoud et al., 2016) while Tavarini et al. (2015) have observed increase in phenolic content up to a certain level of nitrogen dose. Likewise, ascorbic acid content is found to vary with nitrogen treatments in different Brassica crops like cabbage (Roni et al., 2015) and cauliflower (Lisiewska and Kmiecik, 1996). Ascorbic acid being an essential vitamin which needs to be obtained through diet is very important as an antioxidant and for synthesis of collagens L-carnitine and conversion of dopamine to norepinephrine.

There is one report of soluble sugars such as stachyose, raffinose and sucrose in diet causing flatulence in animals and digestive problems in poultry (Hartwig et al., 1997). Since seed meal from mustard has been suggested as a protein source for livestock, aquaculture and also for human consumption (Wanasundara, 2011), it is also necessary to look for potential anti nutritional factors in it. Also more emphasis should be given to soluble proteins than total proteins as they add more value to the seed meal. For example it is important to know the amount of storage proteins like albumin and globulin as they can be used for fortification of food products. Low fiber meal is preferred for animal feed because it increases the digestibility and palatability of the feed. The low metabolizable energy of mustard seed meal (2000 kcal/kg) compared to that of soybean (2230 kcal/kg) is one of the reasons for considering mustard seed meal as inferior (Suprianto, 2014).

Increasing population and dwindling natural resources have promoted the excess use of fertilizers. High yielding varieties of crops also demand heavy use of fertilizers which results in irreversible degradation of soil and natural resources. Excessive use of fertilizers also contributes to global warming. There have been numerous studies that relate fertilizer use and its effect on yield and oil content of oilseed Brassica (Joshi et al., 1998; Parmar and Parmar, 2012). However, little is known about the effect of fertilizer application on chemical composition of oil and seed meal.

Keeping this in view, in this study we have tried to see the effect of nitrogen fertilizer on the nutritional quality of oil and seed meal. In many of the mustard growing regions in India, use of 80 kg N/ha has been recommended to enhance the productivity of mustard (Shekhawat et al., 2012). No data to our knowledge are available on the study of nutritional composition of oil and meal in relation with nitrogen fertilizer application in Indian mustard. Such studies can open new avenues for future research and proper documentation pertaining to Indian mustard.

Materials and methods

Experimental site

The experiment was conducted at ICAR-Directorate of Rapeseed Mustard Research (DRMR) Bharatpur (27°11’N Lat. and 77°27’E Long.); India, during 2013-15. The climate of the region is predominantly semi-arid with long hot summers, intermittent short rainy period due to south-west monsoon and almost 5 month long winter when average temperature drops to below 22.5°C. The winter season often coincides with one or two rains due to western disturbances. The soils of the region are mainly coarse to fine loamy, deep, well drained Inceptisols and fallow-mustard is the predominant cropping system of the region. In general, the soils of the experimental site was alkaline (pH 8.1), low in exchangeable salts (EC 0.6 dS/m), organic carbon content (2.6 g/kg), available N (95.4 kg N/ha $KMnO_4$ oxidisable) and available P (5.4 kg P/ha 0.5N-

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$NaHCO_3$ extractable); and high in available K (267 kg 1N-NH_4OAC exchangeable K_20/ha). Initially, the field was put under exhaustive pearl millet (*Pennisetum glaucum*) – Indian mustard without any external fertilizer inputs for continuous three pre-experimentation years. *Pennisetum glaucum* was sown in the first week of July every year followed by Indian mustard var. Rohini.

Experimental design

During 2013-14, a replicated trial in split plot design keeping two nitrogen environments (0 and 80 kg N/ha) in main plot and 108 oilseed Brassica (OSB) genotypes in sub-plot was conducted to screen out low nitrogen requiring germplasms. Based on field and yield attributes, 24 genotypes were found promising. These selected 24 genotypes were further evaluated for detailed growth, yield and biochemical studies during 2014-15 under two distinct N environments. The genotypes were sown at 5 cm depth in 30 cm row spacing with the help of manual plough in subplots (4.5 m x 5.0 m) during second fortnight of October 2014. The plant to plant distance was maintained at 10-15 cm by thinning at 20-25 days after sowing (DAS). As per the treatment, half dose of nitrogen as urea, full recommended dose of phosphorus in the form of single super phosphate were applied at the time of sowing, and the remaining nitrogen in the form of urea was top dressed after the first irrigation. At maturity, 5 plants were randomly selected from the second and third row from the left side of the plot for recording growth and yield attributes. Harvesting was done by avoiding boarder area and observation lines manually by cutting at surface level using sickles to estimate biological and economic yields. Further, three samples from each plots were collected for all biochemical analysis. Each biochemical parameters for accuracy of data were done in three technical replicates.

Total nitrogen

The total nitrogen was estimated by miro-kjeldahl (Pro-Nitro A Model of JP Selecta, Spain) method as procedure suggested by AOAC (1955).

Fatty acid profiling

Fatty acid profiling was carried out as described by Paquot and Hautfenne, (1987) with little modification. A Nucon model 5765 gas chromatograph with SP-2300 (2%) + SP-2310 (3%) packed silica column was used. The programme was set at N_2 flow rate: 30 ml/min, H_2 flow rate: 30 ml/min, Zero air flow rate: 300 ml/min, Injector temp: 240 ^oC and Detector (FID) temp: 250 ^oC. Individual fatty acids were compared to the retention time of standard methyl esters of fatty acids.

Stability index of oils

Stability index of oils were calculated empirically by the ratio of MUFA: PUFA (Chauhan et al., 2010).

Defattening of seed meal

Oil was extracted from ground seeds after homogenizing with n-hexane using pestle and mortar at room temperature. This was repeated for three to four times to ensure

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complete extraction of oil. The seed meal was dried till hexane is completely removed and stored at 4 ^{o}C.

Total phenols and crude fiber

Total phenols and crude fiber were estimated by non destructive method using FTNIR-Bruker (model Matrix-1) as described by Bala and Singh (2013).

Vitamin C (Ascorbic Acid)

Ascorbic acid was estimated using the titrimetric method described by Ranganna (1986). 0.1g of the defatted seed meal was homogenized with 3% HPO_3 and final volume made to 10 ml. The supernatant was collected into glass vials after centrifugation for 10 min at 5000 rpm. Aliquot of the extract was taken in a 25-50 ml conical flask and titrated against the standardized dye (2,6 -dichlorophenol indophenol) till persistent pink color was detected that lasted for 5s. Vitamin C was calculated using the following formula.

$$\text{mg of ascorbic acid}/100\text{ g} = \frac{\text{Titre } x \text{ Dye Factor } x \text{ Final volume made up}}{\text{Aliquot of extract taken } x \text{ Weight of sample taken}}$$

Total antioxidant capacity

Total antioxidant capacity (TAC) was determined as per Prieto et al. (1999). 100 mg of defatted seed meal was dissolved in 80 % methanol overnight. The supernatant was collected after centrifuging at 4000 rpm for 10 min and final volume was raised to 2 ml. Reduction of Mo(VI) to Mo (V) and the subsequent formation of green colour complex was measured by spectrophotometer (Labomed UV-VIS Double beam UVD-3500) at 695 nm and is expressed as ascorbic acid equivalent (AAE).

Total soluble sugars

Total soluble sugars were estimated as per protocol of Hansen and Møller (1975). 100 mg of defatted seed meal was homogenized with 100% acetone by vortexing. After centrifugation the residue was washed with hot ethanol (80%). This was repeated twice. Supernatant was collected and final volume was raised to 2 ml with 80% ethanol. This extract was used for the estimation of total soluble sugar by Anthrone method whose absorbance was taken at 620 nm.

Total soluble protein

Soluble protein content in the defatted seeds was estimated by the method of Lowry et al. (1951) after precipitation with trichloroacetic acid. Soluble protein was resuspended in laemmili lysis buffer denatured at 95^{0}C for 4 min.

Gel electrophoresis

SDS-PAGE of seed storage protein was carried out in a 12 % Polyacrylamide gel in a discontinuous buffer system. 15 µl (20 µg/ µl) was loaded in each well of the stacking gel. Wide range prestained protein ladder was run as reference. The gel was stained with solution containing of 2 % Coomassie Brilliant Blue, followed by destaining after 1 h with mixture of methanol, acetic acid and water (5:1:4).

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Statistical analysis

The data obtained under different treatments with respect to various parameters were subjected to Analysis of Variance (ANOVA) using SAS 9.3 software package.

Pair-wise comparisons on the least squares mean (LSMEANS) were performed using the Tukey's honest significant difference (HSD) test.

Results and discussion

Application of nitrogen influences the nutritional quality of meal

Total nitrogen content in seed

Studies on the effect of nitrogen on quality traits have been carried out in leaves of *B. juncea* and *B. rapa* (Fallovo et al., 2011), cabbage (Leja et al., 2007), curly kale (Groenbaek et al., 2016) and broccoli (Zaghdoud et al., 2016), but not many reports on effect of applied nitrogen on quality of seed and oil are available. In our study, total seed nitrogen content ranged from 2.86 % (Maya and RL-1359) to 3.95 % (EC399300) at zero nitrogen (N0) application and 3.1% (RL-1359) to 4.3 % (IC212031) at 80 kg N/ha (N80) (*Table 1*). About 63 % of the genotypes showed increase in total seed nitrogen by 3 % (DRMR-IJ-31) to 34.0 % (NRCDR-2) over N0.The increase in total seed N content was proportional to the increase in total soluble protein which varied from 1% (Maya) to 34 % (NRCDR-2), with a confirmation of a positive correlation (r=0.33, p0.019) (*Table 2.a*). Though, exceptions in some genotypes showed reduction in soluble protein by 4 % (78-1-1-1) to 16% (NATP-124 and RGN-55) (*Table 1*). The decrease in soluble protein, even when there is increase in nitrogen intake, could be due to the partition of nitrogen towards the synthesis of other carbon containing compounds in TCA cycle as it shares common substrates. This is substantiated, by increase in ascorbic acid (14. 29 % to 50 %) and phenol (1.14 % to 14 %) content (*Table 1*). However, rest of the 37 % genotypes showed decrease in seed N content at N80 by 5.6 % (HB9916) to 20.2 % (EC399300) over N0. These are the genotypes that also showed a reduction in soluble proteins from 5 % (78-1-1-1, NRCHB-101, HB9916) to 16 % (RGN-55) and could be less responsive to nitrogen uptake. According to Chopin et al. (2007) in *Arabidopsis* there are high and low affinity nitrate transporters that regulate the uptake of nitrogen into plants. Our study also found genotypes such as EC399294 (5%), EC399307 (14%) and GM-2 (7%) that showed an increase in soluble proteins in spite of low content nitrogen. This interplay of nitrogen fertilization × genotype × quality trait needs more in-depth investigation.

Total phenol content

Natural antioxidants and their use as food additives or supplements has been a hot topic in recent years. Members of Brassica family are known to possess health promoting phytochemicals, especially those with antioxidant properties. Among the phytochemicals, phenolic compounds are very important due to their antioxidant properties (Björkman et al., 2011).

The total phenol content in our study ranged from 1.35 % (RL-1359) to 2.26 % (Rohini) under N0 and from 1.46 % (HB-9902) to 2.18 % (DRMR-IJ-31) under N80 (*Table1*). There are reports of decrease in phenol content under higher doses of nitrogen fertilizer (Li et al., 2008, Giorgi et al., 2009; Zaghdoud et al., 2016) which was also

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observed in our study as the phenol content reduced by 0.83 % (QM-16) to 21.83 % (Rohini) in about 33% of genotypes (*Table 1*). This reduction in phenol content as reported by Ibrahim et al. (2011) could be due to inhibition of phenylalanine ammonia lyase (PAL) at higher levels of nitrogen or may be due to diversion of metabolites in the direction of protein synthesis. As expected, 63 % of our genotypes increased in phenol content by 1.14 % (GM-2) to 14.01 % (RL-1359) over N0 application (Table1). Our studies is in tune with work by Groenbaek et al. (2016) in traditional kale. Surprisingly, 4 % of our genotype showed no change in phenol content after N80 application. It is interesting to note within the same species the effect of nitrogen can have varied effects on the phenol content.

This discrepancy between different reports could be due to the differences in the form of nitrogen fertilizers (nitrate or ammoniacal) applied. Fallovo et al. (2011) reported increase in phenol content in the leaves of *B. juncea* and *B. rapa* due to forms of N fertilizer. Leja et al. (2007) observed decrease in concentration of phenolic compounds in cabbage head which were fertilized using calcium nitrate, ammonium sulphate, ammonium nitrate and urea by broadcasting. However, Smolen and Sady (2009) observed rise in phenolic concentration in carrots irrespective of the type and mode of nitrogen application. Sousa et al. (2008) have reported the profile of phenolic compounds to have varied even within the plant organ of the same Brassica species.

Our study suggests that variation in total phenol content is due to genetic variability that determines the optimization of nitrogen uptake and in turn alters the biochemical composition. According to Nguyen and Niemeyer (2008) in basil (*Ocimum basillicum* L.) phenolic content is a genotype dependent property. In this study more number of cultivars were analysed unlike the works reported previously in one or two cultivars.

Vitamin C (Ascorbic acid)

Another important natural antioxidant studied was ascorbic acid, an essential nutrient usually obtained from fruits and vegetables. The biological functions of ascorbic acid include radical scavenging, electron transport in plasma membrane and as cofactor in biosynthesis of collagen and conversion of dopamine to norepinephrine. It can scavenge superoxide and hydroxyl radicals besides regenerating α-tocopherol.

Our study on Indian mustard genotypes, showed significant variation in ascorbic acid content from 43mg/100g to 172 mg/100g seed meal (*Table 1*). However, about 58 % of the genotypes showed ascorbic acid content to increase by 14.29% (HB9916) to 50% (EC399307, NATP-124, NRCDR-2, BEC-16), about 21 % genotypes showed decrease in ascorbic acid content by 20 % (QM-16) to 100 % (HB-9902) and the rest 21 % of the genotypes did not show any change. Increase in ascorbic acid content upon nitrogen treatment has also been reported in cabbage (De and Shankar, 1987). Decrease in ascorbic acid in cabbage (Freyman et al., 1991) and cauliflower (lisiewska and Kmiecik, 1996) have also been reported. The variation in ascorbic acid content could be due to their sensitivity to the enzyme GDP-Mannose pyrophosphorylase, an enzyme essential for the synthesis of metabolite GDP-Mannose, a precursor of ascorbic acid which has been confirmed in model plant to be hypersensitive to NH_4^+ and is a genetic determinant for NH_4^+ sensitivity (Qin et al., 2008). As we have also observed not all genotypes studied had the same levels of nitrogen in seed. This could be the reason for differences in ascorbic acid among the different genotypes. Our study is the first of its kind done in Indian mustard and more enquiries are needed to prove whether there is any dose dependent effect of nitrogen application on ascorbic acid content in seed meal.

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Table 1. *Biochemical Composition of 24 genotypes of B. juncea at N0 and N80 application*

Genotypes	Total nitrogen (%)		Total phenols (%)		Ascorbic acid content (mg/100g)		Total antioxidant capacity (mg/g AAE)		Total soluble sugars (mg/g)		Total crude fiber (%)		Total soluble protein (%)	
	N0	N80	N0	N80	N0	N80	N0	N80	N0	N80	N0	N80	N0	N80
DRMR-IJ-31	3.72^{abcd}	3.85^{abc}	1.93^{a}	2.18^{a}	71.67^{f}	86.00e	39.67^{onp}	46.83^{fgh}	178.33^{q}	133.33^{x}	8.84^{nmopqr}	5.69^{u}	13.97^{w}	16.76^{qprst}
Maya	2.86^{abcd}	3.58^{d}	1.75^{a}	1.71^{a}	107.50^{c}	86.00e	39.83^{onp}	34.33^{rq}	243.33^{g}	223.33^{k}	9.24^{ijklmnop}	8.99^{klmnop}	15.44^{tuvw}	15.59^{stuvw}
78-1-1-1	3.55^{abcd}	3.31^{bcd}	1.72^{a}	1.74^{a}	43.00^{i}	43.00^{i}	45.75^{jihg}	40.00^{on}	143.33^{w}	131.11^{y}	10.3^{defghijklmn}	9.18^{klmnopqr}	17.11^{mnopqrs}	16.42^{qrstu}
DHR-991	3.4^{abcd}	3.58^{abcd}	1.76^{a}	1.675^{a}	64.50^{g}	86.00^{e}	45.50^{jih}	45.00^{jik}	546.67^{a}	404.44^{b}	9.85 ^{efghijklmn}	9.34^{hijklmnop}	15.67^{stuv}	18.43^{ijklmn}
EC399294	3.68^{abcd}	3.12^{bcd}	1.67^{a}	1.83^{a}	43.00^{i}	57.33^{h}	39.83^{onp}	46.00^{fihg}	108.33^{a}	81.11^{f}	8.89 ^{lmnopqr}	10.49^{bcdefghijkl}	21.02^{edf}	22.09^{bdc}
EC399300	3.95^{ab}	3.15b^{cd}	1.76^{a}	1.84^{a}	57.33^{h}	86.00^{e}	47.33^{efg}	51.50^{c}	74.44^{g}	62.22^{i}	9.8^{efghijklmn}	9.59 ^{ghijklmno}	18.65^{hijklm}	21.23^{edc}
EC399307	2.94^{dc}	3.54^{abcd}	1.84^{a}	2.01^{a}	86.00^{e}	172.00^{a}	39.67^{onp}	54.33^{b}	143.33^{w}	60.00^{j}	8.76^{nmnopqr}	6.22 ^{tu}	18.02^{ijklmnopq}	15.92^{rstuv}
GM-2	3.85^{abc}	3.15^{bcd}	1.73^{a}	1.75^{a}	86.00^{e}	86.00^{e}	44.17^{jkl}	42.67^{ml}	276.67^{d}	66.67^{h}	8.17^{opqrs}	8.08^{pqrs}	18.22^{ijklmnop}	19.47^{fghi}
Rohini	3.12^{bcd}	3.97^{ab}	2.26^{a}	1.855^{a}	71.67^{f}	43.00^{i}	48.83^{ef}	50.67^{c}	227.78^{j}	165.56^{s}	7.03^{stu}	7.53^{rst}	17.88^{ijklmnopq}	18.30^{ijklmno}
HB-207	3.64^{abcd}	3.61^{abcd}	1.53^{a}	1.65^{a}	172.00^{a}	129.00^{b}	44.50^{jik}	47.50^{ef}	182.22^{0}	126.67^{z}	9.98^{efghijklm}	10.68^{abcdefghij}	18.96^{hijkl}	21.56^{edc}
HB-9902	3.21^{bcd}	3.66^{abcd}	1.46^{a}	1.46^{a}	86.00^{e}	43.00^{i}	45.00^{jik}	33.33^{rs}	97.22^{b}	60.00^{j}	10.86^{abcdefghi}	10.48^{bcdefghijkl}	17.43^{lmnopqr}	20.66^{edfc}
HB-9912	3.92^{abc}	3.89^{abc}	1.71^{a}	1.76^{a}	43.00^{i}	43.00^{i}	23.83^{vw}	21.83^{yx}	173.33^{r}	35.00^{k}	11.07^{abcdefg}	9.1^{klmnopqr}	19.11^{ghijk}	21.27^{edc}

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HB-9916	3.4^{abcd}	3.21^{bdc}	1.44 ^{a}	1.65 ^{a}	86.00^{e}	100.33^{d}	20.83^{y}	48.67^{e}	277.78^{d}	150.00^{v}	11.64^{abc}	10.62^{abcdefghij}	18.59^{hijklm}	17.60^{jklmnopq}
IC212031	3.21^{bcd}	4.3^{a}	1.66 ^{a}	1.69 ^{a}	86.00^{e}	86.00^{e}	33.17^{rs}	22.50^{wx}	133.33^{x}	90.00^{d}	10.48^{bcdefghijkl}	9.95^{defghijklm}	17.04^{mnopqrst}	18.45^{ijklmn}
NATP-124	3.9^{abc}	3.94^{ab}	1.57 ^{a}	1.65 ^{a}	43.00^{i}	86.00^{e}	24.33^{v}	31.83^{ts}	261.67^{f}	200.00^{m}	11.54^{abcd}	10.82^{abcdefghi}	24.15^{a}	20.07^{efgh}
QM-16	3.36^{abcd}	3.48^{abcd}	1.82 ^{a}	1.805 ^{a}	86.00^{e}	71.67^{f}	39.67^{onp}	23.50^{vw}	237.78^{h}	92.22^{c}	9.81^{efghijklmn}	8.46^{nopqrs}	24.25^{a}	22.84^{abc}
RGN-55	3.68^{abcd}	3.2^{7bcd}	1.68 ^{a}	1.84 ^{a}	71.67^{f}	86.00^{e}	23.33^{vwx}	31.17^{t}	263.33^{e}	133.33^{x}	10.5^{bcdefghijkl}	10.21^{cdefghijklm}	21.01^{edf}	17.54^{klmnopqr}
NRCHB-101	3.67^{abcd}	3.4^{abcd}	1.81 ^{a}	1.80 ^{a}	57.33^{h}	86.00^{e}	30.83^{t}	28.83^{u}	160.00^{u}	80.00^{f}	9.88^{efghijklmn}	9.76^{ghijklmno}	16.89^{nopqrst}	17.79^{jklmnop}
NRCDR-2	3.19^{bcd}	3.88^{abc}	1.72 ^{a}	1.62 ^{a}	43.00^{i}	86.00e	42.50^{m}	43.67^{mkl}	297.78^{c}	96.67^{b}	12.2^{a}	11.2^{6abcdef}	17.40^{lmnopqr}	23.40^{ab}
NRCHB506	3.16^{bcd}	3.28^{bcd}	1.83 ^{a}	1.64 ^{a}	86.00^{e}	86.00e	57.17^{a}	39.83^{onp}	185.56^{n}	162.22^{t}	11.5^{4abcd}	10.94^{5abcdefgh}	17.87^{ijklmnopq}	18.15^{ijklmnop}
Pusa Jai kisan	3.16b^{cd}	3.17^{abcd}	1.56 ^{a}	1.67 ^{a}	57.33^{h}	71.67^{f}	23.67^{vw}	35.17^{q}	163.33^{t}	88.33^{e}	11.32a^{bcdef}	10.52^{bcdefghijk}	14.02^{w}	14.51^{vw}
NDRE-7	3.04^{d}	3.4b^{cd}	1.68 ^{a}	1.58 ^{a}	57.33^{h}	71.67^{f}	35.17^{q}	40.83^{n}	230.00^{i}	173.33^{r}	11.39^{abcde}	11.33^{abcdef}	14.88^{uvw}	17.52^{klmnopqr}
RL-1359	2.86^{bcd}	3.1^{bcd}	1.35 ^{a}	1.57 ^{a}	43.00^{i}	57.33^{h}	38.33^{p}	39.17^{qp}	202.22^{l}	180.00^{p}	12.02^{ab}	11.1^{abcdefg}	15.56^{stuvw}	16.64^{pqrst}
BEC-16	3.08^{bcd}	3.14^{bcd}	1.73 ^{a}	1.92 ^{a}	43.00^{i}	86.00^{e}	38.50^{op}	50.33c^{d}	276.67^{d}	243.33^{g}	8.93^{klmnopqr}	7.57^{rst}	18.18^{ijklmnop}	19.20^{ghij}

T comparison lines for least Squares means of genotypes*nitrogen
LS- means with same letters are not significantly different

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Total antioxidant capacity

Phenolic compounds and ascorbic acid are known for its antioxidant properties which are highly desirable for improving the keeping quality of meal as well as for providing health benefits. Seed meal extracts of 24 genotypes of Indian mustard showed TAC to range between 20 mg/g AAE (HB-9916) to 48 mg/g AAE (Rohini) under N0 and between 21.83 mg/g AAE (HB9912) to 54 mg/g AAE (EC399307) under N80 application (*Table 1*). We observed, 58 % of genotypes showed TAC to increase and 42 % of genotypes showed reduction in TAC over N0. It may be noted that the genotypes in which the TAC was lowered also showed concomitant reduction in ascorbic acid. According to Nguyen and Niemeyer (2008) reduction in TAC upon treatment with NO_3^- correlated with phenol content. Aires et al. (2011) examined the potential antioxidant activities of ascorbic acid, total flavanoid and total phenol in Brassica vegetables and concluded that among the naturally occurring antioxidants, ascorbic acid contributed the maximum towards antioxidant capacity. Our findings are in agreement with this. Ochoa-Velaso et al. (2016) have reported that TAC was strongly influenced by ascorbic acid and phenolic content in tomatoes. The correlation between TAC with that of phenols and ascorbic acid observed in our study (r=0.316, p=0.028) is comparatively less but statistically significant (*Table 2a*).

Table 2a. *Pearson Correlation between total antioxidant, vitamin C and total phenol*

Pearson Correlation Coefficients, N=48 Prob> |r| under H0: Rho=0				
	Total antioxidant capacity	**Phenol**	**Ascorbic acid**	**Phenol+ Ascorbic acid**
Total antioxidant capacity	1.00000	0.36613 0.0105	0.31511 0.0291	0.31699 0.0281
Phenol	0.36613 0.0105	1.00000	0.12944 0.3806	0.13515 0.3597
Ascorbic acid	0.31511 0.0291	0.12944 0.3806	1.00000	0.99998 <.0001
Phenol+ Ascorbic acid	0.31699 0.0281	0.13515 0.3597	0.99998 <.0001	1.00000

Table 2b. *Pearson Correlation between total nitrogen and soluble protein*

Pearson Correlation Coefficients, N= 48 Prob > |r| under H0: Rho=0		
nitrogen	**nitrogen**	**protein**
protein	1.00000	0.33743 0.0190

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Soluble sugars

Seed meal of *B. juncea* is comprised of about 30% carbohydrates which includes many beneficial as well as harmful components. One report showed soluble sugars such as stachyose and raffinose are harmful as it causes flatulence in animals and digestive problems in poultry (Hartwig et al., 1997). In this study, we evaluated the effect of N80 treatment on soluble sugar content. The total soluble sugar ranged between 74.44 mg/g (EC399300) to 546.71 mg/g (DHR-991) under N0. Under, N80 application it ranged from 60 mg/g (EC399307, HB9902) to 404 mg/g (DHR-991). All genotypes reported reduction in total soluble sugar level ranging from 8 % (Maya) to 80% (HB-9912). Similar observations were made by Smolen and Sady (2009) in carrots (*Daucus carota* L.). Further, Krober and Cartter (1962) had shown reduction in soluble sugar content in four cultivars of soybean at higher nitrogen input with a concomitant increase in protein content. Higher nitrogen levels may divert the biosynthetic process as towards the amino acids and proteins consequently reducing the production of soluble carbohydrates. This may be true in 63% genotypes having inverse relationship between soluble sugars and seed nitrogen content. But, contrasting results was observed in 37 % of genotypes where they experienced reduction in both parameters after N80 treatment.

Cruder fiber

Fiber is primarily derived from cell walls and is made up of cellulose, hemicelluloses and lignin. High fiber in animal's diet is undesirable as it would dilute the availability of energy, proteins and minerals such as Mn and Zn (Suprianto, 2014). As an animal feed, the seed meal should be preferably rich in protein and energy rich carbohydrates rather than crude fiber that only add to the bulk and cause digestive disorders. The crude fiber content in the genotypes under N0 ranged between 7.03 % (Rohini) and 12.02 % (RL-1359 and NRCDR-2) whereas; it ranged from 5.69 % (DRMR-IJ-31) to 11.33% (NDRE-7) at N80 application (*Table 1*). 88 % of the genotypes showed reduction in crude fiber content by 1.10 % (GM-2) to 35 % (DRMR-IJ-31) over N0. One report is in agreement with decrease of crude fiber content in fodder beet but it is also dependent on the type of nitrogen fertilizer (Khogali et al., 2011). Within the same experiment they observed no significant effects in maize. Similarly, in forage kale no effect on fiber content was observed (Chakwizira et al., 2015). It is quite evident that response to nitrogen application is not the same in all crops. In the present experiments about 12 % of genotypes showed increase in crude fiber content by 7 % (Rohini, HB-207) to 17.9 % (EC399294). This suggests that variations in seed nitrogen upon N80 application leads to variation in crude fiber and is genotype dependent.

Total soluble protein and seed storage protein

The two major seed storage proteins of oilseed *Brassica* are the 2S albumin called napin and the 11S or cruciferin a legume type globulin having heteromeric structure. Cruciferin, rich in nitrogen containing amino acids have role in modulating nitric oxide signaling which is potential regulator of nutrient metabolism (Wanasundara, 2011), while albumin rich in sulphur containing amino acids have been annotated with several allergic responses such as celiac disease and Baker's asthma (Wanasundara, 2011). However, not all 2S

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albumin causes allergy and it affects only a minority of the population (Wanasundara, 2011). None the less, both storage proteins have found its place in industry as they are highly surface active in monolayers and emulsion formation (Wanasundara, 2011).

Profiling of seed storage proteins with respect to nitrogen treatment has never been done in Indian mustard. We have profiled the seed storage proteins in 24 genotypes of *B. juncea* using SDS–PAGE to see the effect of nitrogen application on the protein profile (*Fig. 1*). Molecular weights of the proteins were compared to previous reports (Wanasundara, 2011, Wanasundara et al., 2012). Our results showed genotypes DRMR-IJ-31, HB-207, HB-9902, NATP-124, RGN-55, NRCHB-506 had low expression of 2S albumin in case of nitrogen treatment (*Fig. 1*). According to Wanasundara et al. (2012) molecular weight of cruciferin ranged between 18.1 kDa and 31.2 kDa in *B.juncea* and that of napin between 6.5 and 12 kDa in reducing environment. But we found the corresponding band between 15 and 16 kDa for napin. This difference in molecular weight may be due to the slight modification in the protocol as we had given heat treatment only for 4 min instead of 15 min as reported earlier. We found genotypic variability in the banding patterns of α and β chains of cruceferin (*Fig 1*). According to Yu (2008) this determines the digestibility of proteins in *B. rapa* and *B. napus.* Since this is the first report in *B. juncea*, it will lead to better ideas on manipulating the meal composition for quality improvement by managing nitrogen fertilization.

Application of nitrogen influences the nutritional quality of oil

Nutritional composition of oil

There are reports on the effect of nitrogen fertilizer on improvement of oil yield and quality (Joshi et al., 1998; Paramar and Paramar, 2012) but, there are no sufficient data to support the role of nitrogen fertilizers in modifying the nutritional composition of oil in *B. juncea* genotypes. Fatty acid profiling in 24 genotypes was done under treated and controlled conditions (*Table 3*). Our results showed, SFA (Palmitic +Stearic acid) content to be within 1.37 % (RGN-55) to 5.76 % (HB-9902) at N0 and between 1.96% (HB-9916) to 4.32 % (NDRE-7) under N80 (*Table 3*). About 50 % of genotypes showed decrease in SFA and 50 % showed increase compared to control, but the rise in SFA levels were still within the permissible limit (< 7%) of healthy edible oil. Paramar and Paramar (2012) have also found a significant decrease in SFA at 50 kg/ha. Reduction in SFA is desirable as it is associated with cardiovascular diseases. Even though, SFA content of Indian mustard is within the optimum range it is possible to manipulate its level by optimizing the nitrogen application as evident from our study.

The level of MUFA (Oleic + Eicosenoic + Erucic) ranged from 50.79% (HB-9902) to 63.51% (NDRE-7) under N0 and from 54.16 % (NDRE-7) to 61.65 % (NATP-124) under N80. The levels of MUFA were found to have increased in about 58 % of genotypes by 1.02% (EC39900) to 20.46 % (HB-9902) over N0, while in the rest 42% of genotypes it decreases by 0.27 % (78-1-1-1) to 14.72 % (NDRE-7) over control.

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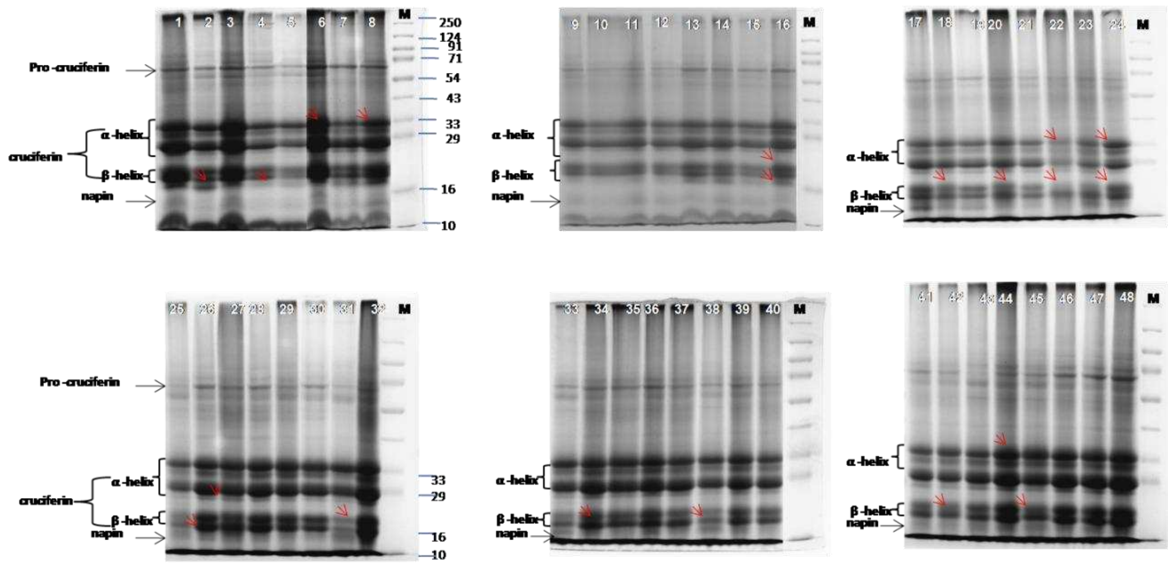

Figure 1. Seed storage protein profiling in 24 genotypes of Indian Musatrd under zero and 80kg/ha nitrogen application

Lane 1 and 2 : DRMR IJ-31 under control and 80kg N/ha; Lane 3 and 4 : Maya under control and 80kg N/ha; Lane 5 and 6: 78-1-1-1 under control and 80kg N/ha; Lane 7 and 8: DHR-991 under control and 80kg N/ha; Lane 9 and 10:GM-2 under control and 80kg N/ha; Lane11and12:EC-399307 under control and 80kg N/ha; Lane13and14: EC-399300 under control and 80kg N/ha; Lane 15and16: EC-399294 under control and 80kg N/ha Lane 17and18: Rohini under control and 80kg N/ha. Lane 19and20:HB-207 under control and 80kg N/ha; Lane 21and22:HB-9902 under control and 80kg N/ha; Lane 23and24: HB-9912 under control and 80kg N/ha; Lane 25and26:HB-9916 under control and 80kg N/ha; Lane 27and28:IC212031 under control and 80kg N/ha; Lane 29and30:NATP-124 under control and 80kg N/ha; Lane 31and32: QM-16 under control and 80kg N/ha; Lane 33and34: RGN-55 under control and 80kg N/ha; Lane 35and36: NRCHB-101 under control and 80kg N/ha; Lane 37and38: NRCDR-2 under control and 80kg N/ha; Lane 39and40: NRCHB-506 under control and 80kg N/ha; Lane 41and42:Pusajai kisan under control and 80kg N/ha; Lane 43and44: NDRE-7 under control and 80kg N/ha; Lane 45and46: RL-1359 under control and 80kg N/ha; Lane 47and48 BC-16: under control and 80kg N/ha; Lane M: wide ranged protein ladder (kDa). (→ indicates the change in α and β banding pattern.

Mustard oil contains moderate levels of PUFA that includes linoleic acid and linolenic acid (15-20 %) which is ideal range for healthy oil. They are essential fatty acids and are required for the biosynthesis of eicosapentanoic acid and docosahexanoic acid, which are beneficial to human beings. In this study, linoleic acid (ω6) ranged from 13.47 % (DHR-991) to 22.94 % (HB-9002) under N0 and 15.20% (NRCHB-506) to 22.38 % (QM-16) for N80. Since, almost all genotypes analyzed in this study are having >15% ω6 content, they can be used as potential source for extraction (Zambiazi et al., 2007). It is to be noted, about 42 % of the genotypes showed increase in ω6 by 1.23% (NRCHB-101) to 29.32 % (DHR-991) over N0. Whereas, 58 % of the genotypes showed reduction in ω6 by 0.89 % (EC399300) to 18.20 % (HB-9916) over N0 application.

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Table 3. *Fatty acid profile of 24 genotypes of B. juncea at N0 and N80 application*

Samples	Palmitic (%)	Stearic (%)	Oleic (%)	Linoleic (%)	Linolenic (%)	Eicosenoic (%)	Erucic (%)	ω6/ω3	SFA (%)	MUFA (%)	PUFA (%)	MUFA:PUFA
DRMRIJ-31 (N_0)	$2.83^{fghijklmn}$	0.17^{qrst}	7.86^{t}	18.7^{at}	19.56^{f}	15.42^{d}	35.15^{x}	0.956^{uvts}	3^{k}	58.43^{s}	$38.26^{fghijklmn}$	$1.53^{bcdefghi}$
DRMRIJ-31 (N_{80})	$2.67^{fghijklmn}$	0.22^{pqr}	8.04^{s}	17.97^{yx}	20.36^{c}	15.29^{e}	34.75^{z}	0.883	2.89^{k}	58.08^{t}	$38.33^{fghijklmn}$	$1.52^{abcdefg}$
Maya (N_0)	3.44^{abcdef}	0.53^{bcd}	9.99^{f}	20.1^{lk}	17.94^{q}	13.26^{l}	34.11^{b}	1.120^{ijklmn}	3.97^{no}	57.36^{w}	38.04^{bcdefg}	$1.51^{bcdefghi}$
Maya (N_{80})	$2.06^{jklmnopqr}$	$0.38^{hijklmn}$	8.72^{n}	17.87^{z}	17.93^{q}	12.2^{u}	39.56^{n}	0.997^{qrstu}	2.44^{x}	60.48^{iw}	35.8^{mnopqr}	$1.69^{abcdefg}$
78-1-1-1 (N_0)	$2.32^{ijklmnop}$	0.24^{pqr}	10.07^{f}	20.92^{g}	19.17^{i}	14.34^{j}	31.75^{f}	$1.091^{jklmnop}$	2.56^{g}	56.16^{x}	$40.09^{ijklmnopq}$	1.40^{fghi}
78-1-1-1 (N_{80})	3.5^{bcdef}	0.21^{qrs}	6.69^{z}	20.54^{i}	17.8^{r}	12.8^{qp}	36.52^{u}	1.154^{ghijk}	3.71^{k}	56.01^{y}	38.34^{bcdef}	$1.46^{bcdefghi}$
DHR-991 (N_0)	2.14^{lmnopq}	$0.41^{fghijklm}$	6.83^{y}	13.47^{g}	13.61^{i}	12.99^{n}	39.64^{nm}	0.990^{qrstu}	2.55^{k}	59.46^{no}	27.08^{lmnopq}	2.20^{a}
DHR-991 (N_{80})	3.52^{bcdef}	0.58	8.42^{p}	17.42^{b}	17.39^{t}	12.01^{v}	40.03^{k}	1.002^{qrst}	4.1^{c}	60.46^{i}	34.81^{bcdef}	$1.74^{bcdefghi}$
EC399294 (N_0)	3.58^{bcdef}	0.48^{defg}	9.97^{f}	18.83^{s}	16.97^{w}	11.85^{wx}	37.63^{q}	$1.110^{ijklmno}$	4.06^{x}	59.45^{o}	35.8^{bcdef}	$1.66^{bcdefghi}$
EC399294 (N_{80})	2.29	0.33^{omn}	8.36^{p}	16.06^{b}	15.97^{az}	10.75^{c}	41.1^{g}	1.006^{pqrst}	2.62^{g}	60.21 j	$32.03^{jklmnop}$	1.88^{bcdef}
EC399300 (N_0)	3.41^{bcdefg}	0.35^{klmn}	9.78^{g}	22.36^{c}	18.45^{nm}	14.93^{g}	30.38^{j}	1.212^{defgh}	3.76^{e}	55.09^{dc}	40.81^{bcdefg}	1.35^{hi}
EC399300 (N_{80})	$3.02^{defghijkl}$	0.48^{dgef}	11.26^{c}	22.16^{d}	17.79^{r}	12.88^{op}	31.51^{g}	1.246^{cdef}	3.5^{h}	55.65^{a}	$39.95^{defhgijklm}$	$1.39^{bcdefghi}$
EC399307 (N_0)	3.79^{bcde}	0.46^{defgh}	9.11^{v}	19.61^{p}	16.73^{x}	13.18^{ml}	36.46^{u}	1.172^{efghij}	4.25^{v}	58.75^{r}	36.34^{bcde}	$1.62^{bcdefghi}$
EC399307 (N_{80})	$2.97^{defghijklmn}$	$0.4^{ghijklm}$	7.8^{tu}	17.61^{a}	15.49^{e}	11.28^{a}	43.25^{d}	1.137^{hijklm}	3.37^{f}	62.33^{c}	$33.1^{defghijklm}$	1.88^{abcdef}
GM-2 (N_0)	$2.97^{defghijkklm}$	0.13^{ts}	6.48^{ao}	18.04^{x}	19.34^{h}	11.94^{v}	41.14^{gf}	0.933	3.1^{t}	59.56^{m}	$37.38^{defghijklm}$	$1.59^{bcdefghi}$

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GM-2 (N_{80})	1.89[nopqr]	0.34[lmn]	8.56[j]	21.47[e]	15.66[c]	7.93[i]	43.74[c]	1.371[ba]	2.23[u]	60.23[kj]	37.13[nopqr]	1.62[abcdefgh]
Rohini(N_0)	1.95[nopqr]	0.19[qrst]	7.52[w]	18.34[v]	19.76[d]	12.12[u]	39.7[1m]	0.928[tuv]	2.14[nm]	59.35[p]	38.1[nopqr]	1.56[bcdefgh]
Rohini(N_{80})	3.1[defghijk]	0.25[opq]	9.47[j]	19.73[o]	15.57[de]	14.57[i]	37.23[r]	1.267[cd]	3.35[z]	61.27[f]	35.3[defghijk]	1.74[bcdefgh]
HB-207 (N_0)	2.84[efghijklmn]	0.22[pqr]	9.66[ih]	20.86	19.81[d]	13.16[m]	32.28[e]	1.053[nmopq]	3.06[f]	55.1[c]	40.67[efghijklmn]	1.35[efghi]
HB-207 (N_{80})	2.17[klmnop]	0.49[cdef]	10.41[e]	19.83[n]	15.06[g]	11.51[z]	40.2[j]	1.317[bc]	2.66 b	62.12[d]	34.89[klmnop]	1.78[bcdefgh]
HB-9902 (N_0)	4.8[a]	0.96[a]	11.87[a]	22.94	18.38[npo]	9.16[f]	29.76[k]	1.248[cdef]	5.76[c]	50.79[i]	41.32[a]	1.23[i]
HB-9902 (N_{80})	2.43[hijklmnop]	0.53[bcd]	8.23[q]	19.65[po]	16.02[z]	11.79[yx]	41.16[gf]	1.227[defg]	2.96[poy]	61.18[g]	35.67[hijklmnop]	1.72[abcdefgh]
HB-9912 (N_0)	4.14[ab]	0.42[efghijkl]	10.4[d]	20.18[k]	19.05[j]	15.13[f]	29.8[k]	1.0591[mnopq]	4.56[i]	55.87[z]	39.23[ba]	1.42[cdefgh]
HB-9912 (N_{80})	3.51[bcdef]	0.5[bdeg]	9.7[hg]	19.62[p]	18.36[po]	11.71[y]	36[w]	1.069[klmnopq]	4.01[ef]	57.41[w]	37.98[bcdef]	1.51a[bcdefgh]
HB-9916 (N_0)	3.23[bcdefghij]	0.53[bcd]	9.14[e]	19.23[r]	16.12[y]	12.75[qr]	38.96[p]	1.193[defghi]	3.76[z]	60.85[h]	35.35[bcdefgh]	1.72[abcdefg]
HB-9916 (N_{80})	1.6[pqor]	0.36[jklmn]	7.74[uv]	15.73[e]	13.31[j]	11.24[a]	41.21[f]	1.182[defghi]	1.96[j]	60.19[k]	29.04[pqor]	2.07[abcd]
IC212031 (N_0)	3.07[defhijkl]	0.37[ijklmn]	9.08[l]	19.42[q]	18.48[m]	14.12[k]	35.28[x]	1.051[mnopq]	3.44[pq]	58.48[s]	37.9[cdefghijk]	1.54[bcdefghi]
IC212031 (N_{80})	3.13[efghid]	0.3[nop]	8.89[m]	20.64[h]	20.49[b]	15.83[c]	30.55[i]	1.007[pqrst]	3.43[p]	55.27[b]	41.13[defgh]	1.34[defghi]
NATP-124 (N_0)	2.93[cdefghijkm]	0.11[t]	7.79[ut]	18.71[t]	19.47[g]	12.7[sr]	37.22[r]	0.961[rstuv]	3.04[lm]	57.71[u]	38.18[defghhijklm]	1.51[abcdefghi]
NATP-124 (N_{80})	3.18[defghi]	0.36[jklmn]	6.98[x]	17.60[a]	17.08[v]	10.24[d]	44.43[a]	1.030[opqrs]	3.54[p]	61.65[e]	34.68[defghij]	1.7[8abc]
QM-16 (N_0)	2.75[fghijklmn]	0.44[jefghi]	10.95[d]	22.70[b]	18.48[m]	12.8[qp]	31.26[h]	1.228[bfge]	3.19[d]	55.01[pe]	41.18[fghijklmn]	1.34[efghi]
QM-16 (N_{80})	3.42[bcdefg]	0.40[ghijklm]	10.49[e]	22.38[c]	19.65[e]	14.78[h]	28.26[l]	1.139[hijkl]	3.82[b]	53.53[h]	42.03[bcdefg]	1.27[bcdefghi]

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RGN-55(N_0)	1.21^{qr}	0.16^{rts}	6.22^{b}	21.04^{f}	22.61^{a}	17.3^{a}	31.44^{g}	0.931^{tuv}	1.37^{a}	54.96^{e}	43.65^{qr}	1.26^{efghi}
RGN-55(N_{80})	3.61^{bcdef}	0.46^{defgh}	8.18^{q}	20.041^{m}	15.93^{a}	11.05^{b}	40.31^{i}	1.258^{dc}	4.07^{w}	59.54^{nm}	35.97^{bcdef}	1.66^{efghi}
NRCHB-101 (N_0)	2.54^{ghijklmno}	0.42^{efghijkl}	9.5^{j}	17.92^{zy}	19.44^{g}	14.07^{k}	34.84^{z}	0.922^{tuv}	2.96^{t}	58.41^{s}	37.36^{ghijklmno}	1.56^{bcdefghi}
NRCHB-101 (N_{80})	3.24^{bcdefghij}	0.41^{fghijklm}	8.12^{sr}	18.14^{w}	17.73^{r}	12.89^{op}	39.28^{o}	1.023^{opqrs}	3.65^{x}	60.29^{j}	35.87^{bcdefghi}	1.68^{bcdefgh}
NRCDR-2 (N_0)	1.52^{pqr}	0.45^{defghi}	11.38^{b}	18.34^{v}	15.63^{dc}	8.87^{g}	43.77^{cb}	1.173^{efghij}	1.97^{e}	64.02^{a}	33.97^{poq}	1.88^{ab}
NRCDR-2 (N_{80})	3.88^{abcd}	0.4^{ahijklm}	11.32^{bc}	20.42^{j}	17.46^{ts}	12.33^{t}	33.9^{c}	1.170^{fghij}	4.28^{q}	57.55^{v}	37.88^{abcd}	1.52^{bcdefgh}
NRCHB506 (N_0)	4.13^{abc}	0.98^{a}	9.5$^{1\ j}$	15.20^{f}	15.83^{b}	12.62^{s}	37.09^{s}	0.960^{stuv}	5.11^{h}	59.22^{q}	31.03^{abc}	1.91^{abcdef}
NRCHB506 (N_{80})	1.7^{defghijklmnopqr}	0.57^{b}	7.49^{w}	15.74^{e}	15.16^{f}	10.77^{c}	36.52^{u}	1.038^{nopqrs}	2.27^{i}	54.78^{f}	30.9^{opqr}	1.77^{abcdef}
Pusa Jai kisan (N_0)	2.93^{defghijk}	0.23^{pqr}	8.57^{o}	19.99^{m}	18.96^{k}	14.36^{j}	34.48^{a}	1.054^{klmnopq}	3.16^{j}	57.41^{w}	38.95^{bcdefghijklm}	1.4^{7hig}
Pusa Jai kisan (N_{80})	3.37^{bcdefghq}	0.47^{defg}	7.74^{uv}	18.75^{ts}	17.53^{s}	11.49^{z}	40.52^{h}	1.070^{klmnopq}	3.84^{v}	59.75^{l}	36.28^{dbcdefgh}	1.65^{bcdefghi}
NDRE-7 (N_0)	1.18^{r}	0.23^{pqr}	6.45^{a}	16.69^{c}	18.35^{p}	13.22^{ml}	43.84^{b}	0.91^{uv}	1.41^{a}	63.51^{b}	35.04^{R}	1.81^{bcdefg}
NDRE-7 (N_{80})	3.34^{bcdefgh}	0.98^{a}	9.47^{j}	20.37^{j}	14.24^{h}	8.45^{h}	36.24^{v}	1.430^{a}	4.32^{d}	54.16^{g}	34.61^{bcdefgh}	1.56^{bcdefg}
RL-1359 (N_0)	3.25^{bcdefghi}	0.13^{ts}	7.64^{v}	17.69^{a}	17.25^{u}	11.94^{wv}	42.07^{e}	1.026^{opqrs}	3.38^{b}	61.65^{e}	34.94^{bcdefgh}	1.76^{abcde}
RL-1359 (N_{80})	3.62^{bcdef}	0.43^{efghijk}	8.84^{m}	18.46^{u}	18.59^{l}	12.9^{on}	36.72^{t}	0.993^{qrstu}	4.05^{u}	58.46^{s}	37.05^{fbec}	1.58^{abcdefgh}
BEC-16 (N_0)	2.7^{fghijklmn}	0.38^{hijklmn}	9.56^{ij}	18.16^{w}	19.44^{g}	9.91^{e}	39.81^{l}	0.934^{uvt}	3.08^{s}	59.28^{qp}	37.6^{fghijklm}	1.58^{bcdefg}
BEC-16 (N_{80})	3.37^{bcdefgh}	0.4^{ghijklm}	9.26^{k}	19.32^{r}	18.44^{nmo}	15.95^{b}	33.22^{d}	1.048^{nopqr}	3.77^{r}	58.43^{s}	37.76^{bcdefg}	1.55^{bcdefg}

T comparison lines for least Squares means of genotypes*nitrogen
LS- means with same letters are not significantly different

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The ω3 levels were found to range between 13.62 % (DHR-991) and 22.61 % (RGN-55) under N0 while, under N80 it ranged between 13.31 % (HB-9916) to 20.49 % (IC212031). These levels are high enough (>12%) to be considered as a source for commercial extraction of ω3 according to Zambiazi et al. (2007) who reported the bench mark to be 12 %. It has been reported earlier that a dose of 60 kg/ha nitrogen fertilizer was optimum to enhance ω3 in Indian mustard (Joshi et al., 1998) but it is contradictory to our findings where we found a variation within the same species at N80. 29 % of the genotypes showed an increase in ω3 by 4.09 % (DRMR-IJ-31) to 27.77 % (DHR-991) over N0 and 71 % of the genotypes reported reduction by 0.06 % (Maya) to 29.54 % (RGN-55) over N0. Previous works have reported the effect of nitrogen only in one genotype and comparative studies have not been explored.

Generally *B. juncea* is known for having all the fatty acids in the ideal range for e.g. high levels of MUFA, low SFA, moderate PUFA, ω6:ω3 ratio of 1.2:1 (Chauhan et al., 2010). Under N0, the ω6:ω3 ranged from 0.91 (NDRE-7) to 1.23 (QM-16) and under N80, it ranged from 0.88 (DRMR-IJ31) to 1.37 (GM-2) (*Table 3*). According to various medical researchers (Simopoulos, 2012) there is so no ideal ω6:ω3 ratio that would prevent chronological diseases. The optimal ratios vary with the disease under consideration and according to their documentation, ratio beyond 10:1 was observed to have deleterious effects on health (Simopoulos, 2012). However, a low level ω6:ω3 is preferable. It was observed that under N80, 50 % of genotypes had increased the ratio by 0.88 % (HB-9912) to 57.28 % (NDRE-7) over N0. While, 50 % genotypes showed reduction in the ratio by 0.33 % (NRCDR-2) to 11.04 % (Maya) over N0.

The MUFA: PUFA ratio depicting oil stability index (OSI) ranged from 1.23 (HB-9902) to 2.20 (DHR-991) under N0 and from 1.27 (QM-16) to 2.07 (HB-9916) in N80 (*Table 3*). Most of the genotypes (96%) failed to reach the ideal range of 2 (Chauhan et al., 2010). However, under N80 about 63 % of genotypes improved in OSI by 1.81 % (GM-2) to 39.54 % (HB9902) while, the rest 37 % of the genotypes showed reduction by 0.78 % (DRMR-IJ-31) to 20.90 % (DHR-991) over N0 application.

Nutritionally undesirable erucic acid was found to exceed the recommended percentage of 2% at both N0 and N80 application (*Table 3)*. Erucic acid, under N80 observed an increase in 63 % of genotypes by 0.9 % (DHR-991) to 38.31% (HB9902) and 37 % of genotypes showed decrease by 1.14 % (DRMR-IJ 31) to 22.5 % (NRCDR-2) over N0. The variations in nitrogen levels hence contribute to the variation in oil quality as seen in case of composition of individual fatty acids.

Conclusion

This study showed N application can bring about changes in oil and seed meal quality. 63 % of the genotypes showed increase in total N content in seeds by 3 % (DRMR-IJ-31) to 34.0 % (NRCDR-2) and rest 37 % genotypes showed reduction in seed N content by 5.6 % (HB9916) to 20.2 % (EC399300) over control. These genotypes also have positive correlation to the total soluble protein content. However, some genotypes showed an inverse relationship between N and soluble protein which could be due to the flux in N to biosynthesis of other carbon compounds as observed by an increase in the case of ascorbic

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acid content and phenol content. Ascorbic acid and total phenolic content showed a positive correlation with TAC (r=0.316, p0.028). Crude fiber and total soluble sugars were observed to decrease under N80. These reductions are desirable for increasing the digestibility and palatability of meal. Seed storage protein profiled under N80 revealed differences in the banding patterns especially in α and β chain of the cruciferin which determines the digestibility and quality of seed meal. Fatty acid profiling showed reduction of SFA and ω6:ω3 in 50 % of genotypes and 50 % of genotypes showed increase in their ratios after N treatment. 63% of genotypes showed improvement in OSI after N80 treatment. N80 application caused 63 % of the Indian mustard genotypes to increase in erucic acid. Our findings showed application of nitrogen even with recommended dose can influence biochemical changes within same species. All in all our observations suggest that nitrogen fertilizers can play a role in enhancing nutritive status of oilseed Brassica when applied in appropriate doses. Information on the effect of nitrogen application on nutritional status of oil and meal will be quite useful in quality improvement programmes.

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ENERGY AND CO_2 EMISSION ASSESSMENT OF WHEAT (*TRITICUM AESTIVUM* L.) PRODUCTION SCENARIOS IN CENTRAL AREAS OF MAZANDARAN PROVINCE, IRAN

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Abstract. Climate change is a global concern and part of it is due to agricultural activities. Therefore, optimizing the agricultural operation is seen as a strategy to alleviate climate change effects. The purpose of this research was energy and carbon dioxide emission assessment in wheat production. To do so, first, six conventional wheat planting fields were identified in the counties of Jouybar, Ghaemshahr and Sari in Iran, in 2015. Then, through improved planting methods (based on growing low-input wheat, designed by researchers) they were compared. The identification of the field was done in a way that covered all main production methods in each county. After that, the improved and conventional methods of planting in the three counties were investigated as four scenarios. The results showed that the average input energy in four scenarios was 11811.61 MJ ha^{-1} where the least input energy in the improved scenario was 11169.72 MJ ha^{-1}. The highest portion of input energy in the four scenarios was of nitrogen fertilizer with average of 4492.14 MJ ha^{-1} and 38.03 per cent of it had the highest rate of CO_2 emission and global warming potential (GWP). Fuel, phosphorus fertilizer, and seed held the next rank of CO_2 emission. The average GWP of wheat production in the four scenarios was 894.07 kg CO_2 per ha^{-1}. The lowest and highest amount of GWP were 710.2 and 933.59 kg CO_2 per ha^{-1} in scenarios I and II, respectively. Moreover, the amount of GWP per energy input unit was the maximum in scenario II and minimum in scenario IV. Furthermore, the improved scenario had the lowest GWP per energy output unit and scenario I achieved the first rank. In general, the amount of GWP has a direct relationship with the method of field management and input consumption.
Keywords: *climate change, cropping system, environment, food security, global warming potential*

Introduction

Taking a look at the statistics and information about the gap between energy consumption in Iran and developed countries, we can see the deficiencies in energy consumption in Iran more clearly. The most important reasons for this are inefficient energy conversion technologies and improper culture of energy consumption. Agricultural production in the 21st century has to go along and result in increased food security and it has to be less dependent on fairly rare resources such as agricultural lands, water, fossil fuel and non-renewable energies (Uphoff, 2012). While modern methods in this area have not been understood completely and are transitioning, their agricultural and scientific justification is being slowly understood. Many achievements have been recorded by making changes and managing plant's growth environment, soil, water and nutrients, and there is a need for more attention from researchers, policymakers and authorities especially for decreasing climate change consequences (Uphoff, 2012). In producing agricultural crops, such as wheat and to do agricultural

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operations such as ploughing, applying fertilizers, pesticides, planting, irrigation, harvesting, processing and transformation, there is need for some forms of energy (Chauhan et al., 2006). Efficient use of energy in agriculture is an important factor for the development of sustainable agriculture because it brings economic saving, preservation of fossil fuel and reduction in air pollution (Pervanchon et al., 2002). Concerns about preserving fossil fuels, and greenhouse gases emission have led to an increase in studies about energy efficiency in crop production systems (Koga, 2008). Global warming, due to greenhouse gases emission, is one of the most important global environmental challenges. It is endangering the future life on earth (IPCC, 2007a). Agriculture has a considerable role in greenhouse gases emission and, consequently, global warming (Robertson et al., 2000). Reducing the fossil energy consumption in agricultural systems can decrease the consumption of the limited energy resources and contribute to reduction of greenhouse gases emissions (Dalgaard et al., 2001).

In a study to investigate the energy input in wheat production in Gorgan region, Iran, it was observed that among all the direct energy inputs, fuel consumed in agricultural operation had the highest place with an average of 3390 MJ ha^{-1} and the second slot was for supplying electricity with an average of 309 MJ ha^{-1} (Soltani et al., 2013). Tipi et al. (2009), by investigating energy consumption in 97 fields in Marmara state in Turkey, show that wheat production consumes 20653.5 MJ.ha^{-1} among which the fuel energy input has the highest portion of total energy consumption with 45.15 per cent, and after that comes the chemical fertilizer with 34.21 per cent (especially nitrogen fertilizer with 31.77 per cent). By analyzing the energy efficiency in the Mediterranean agriculture systems in a research, it was reported that energy consumption of low input systems had significantly decreased to 30 per cent. The most important input resources in canola production were chemical fertilizers (64.66 per cent), diesel fuel (24.45 per cent) and pesticides 4.14 per cent (Nassi et al., 2011). Furthermore, in a study done by Ghorbani et al. (2011), the amounts of energy input in the low input and high input planting system for wheat were 9354.2 and 45367.6 MJ ha^{-1}, respectively. Koocheki et al. (2011), while investigating the total energy needed in the fields of bean *(Phaseolus vulgaris* L.*)*, lentil *(Lens culiparis* L.*)* and pea *(Cicer arietinum* L.*)* states that the energy inputs in bean and lentil fields were 23666.8 and 14114.79 MJ ha^{-1}, respectively. Also, in the irrigated and rain-fed cultivation of pea, energy input was reported to be 15756.21 MJ ha^{-1} and 2630.12 MJ ha^{-1}, respectively. In studying 13 scenarios of sugar beet production in England, the average of the total GWP was obtained to be 1.25 t eq-CO_2 ha^{-1}. Also the average GWP for production was estimated to be 0.024 t eq-CO_2 t^{-1} which was 0.0062 t eq-CO_2 GJ^{-1} per unit energy output. According to various production conditions in each of the scenarios, it was said that the amount of GWP had a direct relationship with the amount of energy input in sugar beet production (Tzilivakis et al., 2005a). The development of agricultural systems with less input and more efficiency can contribute to reducing CO_2 emission in agricultural section. Agriculture, especially growing wheat, is considered a noticeable factor in the release of greenhouse gases. It is necessary to investigate the wheat production cycle to determine the amount of energy consumed in order to reduce greenhouse gas emission and global warming. Therefore, the aim of this research was to investigate the energy consumption and global warming potential in wheat production fields in the central parts of Mazandaran province in Iran.

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Materials and methods

Description of the site

Mazandaran province is located in the north of Iran. The experimental region is geographically situated at 35°, 47' till 36°, 35' N latitude and 50°, 34' till 54°, 10' E longitude. Based on temperature, rain, and topography of the region, this province is divided into two climates of Caspian mild weather and mountain weather. This research was done in the Caspian mild weather comprising the central portion of the province to the northern foothills. As a result of being close to the Caspian Sea on one side and the Alborz mountain range on the other and due to the short distance between the sea and the mountain, this region enjoys a mild temperature which leads to considerable rain. The mean annual rain in the coastal area of the province is 977 mm. The maximum rainfall occurs in fall and the minimum in spring. Hot and humid summers and mild and humid winters are the main characteristics of this type of weather. Therefore, the weather in some parts of this area is similar to that of the Mediterranean. Also, soil properties in each wheat production scenarios in different counties in depth of 0-30 cm are detailed in *Table 1*.

Table 1. *Soil properties in each wheat production scenarios in different counties (0-30 cm)*

Item		Soil texture	EC (dSm^{-1})	pH	Organic matter (%)	P (ppm)	K (ppm)
Scenario I	Field 1	CL	0.64	7.73	1.92	8.2	160
	Field 2	CLS	1.01	7.63	2.04	15.8	174
	Field 3	LCL	0.51	7.76	1.41	15.4	197
	Field 4	SCL	0.45	7.63	1.66	9.2	246
	Field 5	LCL	0.35	7.72	2.11	19.5	401
	Field 6	L	1.40	7.6	4.23	10.5	464
Scenario II	Field 1	SL	0.61	7.62	2.04	13.1	391
	Field 2	C	0.55	7.71	1.34	5.6	234
	Field 3	CS	1.46	7.68	1.81	10.1	219
	Field 4	CS	0.56	6.55	2.61	11.3	220
	Field 5	C	0.52	7.46	2.11	16.3	452
	Field 6	CL	0.50	7.54	2.36	19.9	490
Scenario III	Field 1	CL	0.44	7.75	1.72	6.9	197
	Field 2	C	0.51	7.66	2.04	5.5	238
	Field 3	SCL	0.52	7.65	2.56	6.1	246
	Field 4	CL	0.39	7.77	1.41	11.4	145
	Field 5	CL	0.47	7.72	1.53	12	160
	Field 6	C	0.53	7.40	1.15	4.9	167
Scenario IV		SC	0.66	7.65	2.87	7.2	221

Description of regions and counties under study

The area this research covers includes three central counties of Mazandaran province (Jouybar, Sari, and Ghaemshahr counties), and based on the research method, we tried to investigate the target population through statistics and scientific methods. To do the research, first, 6 fields for conventional planting of wheat for each county were identified in 2015. Then, they were compared to the improved planting method (according to the growing low input wheat developed by the researchers). Identification of the fields was done in a way that covered all main production methods in every county. After that, the improved and conventional methods of planting in the three counties were investigated as four scenarios. The features of the fields and their complementary information are presented in *Table 2*.

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Table 2. *Description of each wheat production scenarios in different counties*

Item		Geographical coordinate	Field area (m^2)	Previous crop	Cultivar	Plow (0-30 cm)	Sowing	Base fertilizer	Top dressing 1	Top dressing 2	Harvest
Scenario I	Field 1	0668661/4052568	30000	Wheat	Zagros & Morvarid	13 Nov.	14 Nov.	14 Nov.	13 Mar.	-	13 Jun.
	Field 2	0607173/4061426	10000	Soybean	Morvarid	25 Nov.	27 Nov.	27 Nov.	28 Feb.	6 Apr.	16 Jun.
	Field 3	0669848/4050342	17000	Wheat	Milan	22 Oct.	15 Nov.	15 Nov.	4 Mar.	-	13 Jun.
	Field 4	0666675/4051164	50000	Soybean	Morvarid	19 Nov.	20 Nov.	20 Nov.	22 Feb.	2 Mar.	15 Jun.
	Field 5	0670958/4050633	15000	Wheat	N-80-19	19 Nov.	20 Nov.	20 Nov.	27 Jan.	28 Feb	13 Jun.
	Field 6	0667482/4061342	20000	Wheat	N-87-20	5 Nov.	10 Nov.	10 Nov.	25 Dec.	7 Mar.	14 Jun.
Scenario II	Field 1	0691941/4069600	28000	Soybean	Milan	8 Nov.	10 Nov.	10 Nov.	20 Jan.	26 Mar.	9 Jun.
	Field 2	0690357/4023752	20000	Canola	Morvarid	20 Oct.	6 Dec.	6 Dec.	11 Apr.	-	15 Jun.
	Field 3	0686313/4068679	20000	Soybean	Morvarid	12 Nov.	14 Nov.	14 Nov.	20 Feb.	10 Mar.	11 Jun.
	Field 4	0709467/4020112	12000	Wheat	Shanghai	14 Nov.	8 Dec.	8 Dec.	22 Mar.	-	25 Jun.
	Field 5	0609483/4024139	10000	Tobacco	N-80-19	27 Nov.	13 Nov.	13 Nov.	-	-	13 Jun.
	Field 6	0702365/4004337	25000	Wheat	Morvarid	22 Oct.	15 Nov.	15 Nov.	-	-	19 Jun.
Scenario III	Field 1	0670166/4047796	18000	Soybean	Morvarid	21 Oct.	20 Oct.	20 Oct.	-	-	9 Jun.
	Field 2	0670429/4044302	12000	Wheat	Milan	28 Oct.	7 Nov.	7 Nov.	12 Mar.	-	5 Jun.
	Field 3	0669674/4042423	7000	Wheat	Morvarid	26 Oct.	9 Nov.	9 Dec.	-	-	10 Jun.
	Field 4	0667144/4040329	7000	Wheat	Milan	25 Oct.	18 Nov.	18 Nov.	4 Mar.	-	3 Jun.
	Field 5	0677217/4038681	10000	Wheat	Milan	25 Oct.	1 Dec.	1 Dec.	20 Feb.	-	11 Jun.
	Field 6	0677851/4037577	20000	Wheat	Shanghai	20 Oct.	21 Nov.	20 Nov.	13 Feb.	-	16 Jun.
Scenario IV		0668497/4051722	8400	Wheat	Milan & Morvarid	20 Nov.	21 Nov.	20 Nov.	20 Feb.	12 Mar.	8 Jun.

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All the managerial practices of the chosen fields were being observed by agriculture engineers. In order to collect information from the fields, first, all agricultural practices were divided into eight parts of providing field, planting, fertilizing, preserving the plants, controlling the weeds, irrigating, harvesting, and transportation. Then, with the beginning of every operation, according to temperature fluctuations, information on various production methods and different amounts of input use by farmers of the region was collected. Typical information of agricultural operations such as the commencement date of every operation and the amount of inputs in every stage of work (from planting to harvesting) was collected and recorded by the observers who went to the fields and observed. Moreover, in the improved planting method, the researchers were seeking reduction of input use, environmental damage and also increase in efficiency and its comparison with common methods of planting wheat in the region. The variables investigated in the improved planting method were as follows:

Improved planting method: The operation of field preparation was done only once by the disc with Massey Ferguson 2850 tractor. The planting operation was done by using 200 kg seed ha^{-1} only by the second disc. The varieties of wheat seeds Milan and Morvarid were planted for dry land farming. According to soil analysis, chemical fertilizers N, P and K (92 kg N h^{-1}, 50 kg P_2O_5 h^{-1} and 50 kg K_2O h^{-1}) were applied to the basal. Moreover, 33.33% N was used in basal, 33.33 % N was used in tillering stage and 33.33 % top dressing of N fertilizer was applied in flowering stage. To control the weeds, herbicide Tapic was applied at 1 litre per hectare once on narrow leaf weeds and simultaneously herbicide Granestar at 25 gram per hectare was applied on wide leaf weeds. Protection operation for fighting pests and diseases was done according to recommendations of the region.

Data collection

All the managerial operation in this is research from the primary plowing and preparing soil to harvesting were recorded through field studies. In these investigations, the method of each managerial operation in the fields was determined in each of the phases of preparing soil, planting, cultivating and harvesting. All data about agricultural management including soil preparation (time and number of plowing, disc, etc.), planting time, fertilizer (amount and time of the applied fertilizer), pests, diseases and weeds control, irrigation (number and time of irrigation) and issues about harvesting (harvest time and yield) were collected. At the end of the growing season, the amount of real harvested yield was recorded. To estimate energy consumption and CO_2 emission, the fields under study in each county with improved planting method were taken as scenarios and totally four scenarios were investigated. To estimate the energy of the inputs and agronomic practices, expressed in MJ ha^{-1}. The energy equivalents in *Table 3* were utilized.

All the information about CO_2 emissions due to direct and indirect energy consumption was recorded and collected. After calculating the amount of energy consumed in the experiment, the result got generalized to an area of one hectare. To estimate energy consumption, the amounts of inputs and outputs were determined. To assess the energy input (consumed), all inputs at the time of practicing the agricultural operations changed into their equivalent by using the relationships of energy equivalence (conversion coefficients) extracted from various sources of every agricultural operation. Then, the input energy for each input and operation was calculated (Singh et al., 2007; Soltani et al., 2013). To determine the energy output

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(produced) obtained from grain and straw, they were changed to their equivalents by using energy equivalence (conversion coefficients) extracted from wheat grain and straw. After that, the total amounts of their input and output energies were calculated separately (Singh et al., 2007; Soltani et al., 2013). With calculation of the total input and output energies, different forms of energy consumption were identified. Direct energy includes fuel and labor. Indirect energy includes seed, fertilizer, pesticides, water and machinery. Moreover, renewable energy includes labor, seed and water used in irrigation. Non-renewable energy includes fuel, fertilizer, pesticides and machinery (Hatirli et al., 2006; Jarvis, 2000; Mandal et al., 2002; Mirin et al., 2001).

Table 3. *Energy content of inputs and outputs. aIncludes energy required for manufacture, repair and maintenance and transportation of machines, ba.i. represents active ingredient*

Inputs	Unit	Energy (MJ/unit)	Reference
Human labor	h	1.96	Johnson et al. (2007)
Wheat seed	kg	15.7	Canakci et al. (2005); Ozkan et al. (2004)
Machinery [a]	h	62.7	Canakci et al. (2005)
N fertilizer	kg N	60.6	Ozkan et al. 2004; Akcaoz et al. 2009
P fertilizer	kg P_2O_5	11.1	Ozkan et al. 2004; Akcaoz et al. 2009
K fertilizer	kg K_2O	6.7	Ozkan et al. 2004; Akcaoz et al. 2009
Diesel	L	38	Soltani et al. (2013)
Electricity	kWh	12.1	Kaltsas et al. (2007)
Insecticide	kg a.i.[b]	237	Rathke and Diepenbrock (2006); Tzilivakis et al. (2005a)
Fungicide	kg a.i.	99	Strapatsa et al. (2001)
Herbicide	kg a.i.	287	Rathke and Diepenbrock (2006); Tzilivakis et al. (2005a)
Outputs			
Wheat grain	kg	14.7	Tipi et al. (2009); Singh et al. (2003)
Wheat straw	kg	9.25	Tabatabaeefar et al. (2009)

Energy assessment indices including energy ratio, energy productivity, specific energy, and net energy yield were calculated for each planting method (Soltani et al., 2013). It has to be mentioned that the described indices have been determined in order to assess the relationship between the total input and output energies which vary according to the type of product, type of soil, the nature of plowing operation for preparing soil, type and amount of chemical fertilizers and manure, cultivation operation, harvest and finally the yield levels (Soltani et al., 2013). The equations of energy indices are:

$$ER=EO/EI \quad \text{(Eq.1)}$$

where:
ER is the energy ratio and is a number without a unit, EO is the total energy output from the field (MJ ha^{-1}), and EI the total energy input to the field.

$$EP=GY/EI \quad \text{(Eq.2)}$$

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where:
EP is the energy productivity (kg MJ^{-1}), GY is grain yield (kg ha^{-1}) and EI the total energy input to the field (MJ ha^{-1}).

$$SE=EI/GY \quad \text{(Eq.3)}$$

where:
SE is the special energy (MJ kg), EI is the total energy input to the field (MJ ha^{-1}), and GY the grain yield (kg ha^{-1}).

$$NEY=EO-EI \quad \text{(Eq.4)}$$

where:
NEY is the net energy yield (MJ ha^{-1}), EO is the total energy output from the field (MJ ha^{-1}), and EI is the total energy input to the field (MJ ha^{-1}).

In order to calculate the global warming potential, first, the fuel for consumption in the factory and the energy consumed for production and transportation of inputs including chemical fertilizers, pesticide, machinery and fuel consumption for agricultural operations were determined (Green, 1987; IPCC, 2007b, 2007c; Tzilivakis et al., 2005a, 2005b). Then, CO_2 emission for every section was calculated. To calculate carbon dioxide emission from winnowing, disinfection and transportation of the grains, the factor was determined according to the type of agricultural management and grain quality (IPCC, 2007a, 2007d). After calculating the total GWP, estimation of CO_2 emission per unit area (kg eq-CO_2 ha^{-1}), per unit weight (kg eq-CO_2 t^{-1}), per unit of energy input (kg eq-CO_2 GJ^{-1}) and per unit of energy output (kg eq-CO_2 GJ^{-1}) were done (Soltani et al., 2013).

Results

Analysis of energy input and energy output

The mean for total energy input in the four scenarios was 11811.61 MJ ha^{-1}. The lowest energy input was obtained in the improved planting method at 11169.72 MJ ha^{-1}, and scenarios II and III got the next rank with little difference (11261.26 and 11262.21 MJ ha^{-1}, respectively). Scenario I, which is the conventional planting method in Jouybar county, bagged the first place with significant and noticeable difference with 13553.25 MJ ha^{-1} *(Table 4).*

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***Table 4.** Energy balance (MJ ha^{-1}) for each wheat production scenario. * Scenario IV is improved planting method. Scenarios I, II, and III are conventional method*

Item	Scenario								Mean	Standard error	Share (%)
	I	Share (%)	II	Share (%)	III	Share (%)	IV*	Share (%)			
Input											
Seed	2820.6	21.81	3359.8	29.84	2862.58	25.42	3140	28.11	3045.75	126.41	25.79
Labor	24.65	0.18	20.09	0.18	30.97	0.27	26.42	0.24	25.53	2.25	0.22
Machinery	614.8	4.54	462.73	4.11	600.67	5.33	366.80	3.28	511.25	59.12	4.33
Fuel	3899.7	28.77	3250.14	28.86	3937.94	34.97	1990.44	17.82	3069.56	645.94	25.99
Chemical fertilizer											
N	5358.2	39.53	3561.87	31.63	3473.29	30.84	5575.2	49.91	4492.14	564.69	38.03
P_2O_5	557.4	4.11	455.67	4.05	324.79	2.88	510.6	4.57	462.12	50.27	3.91
K_2O	191.3	1.41	124.82	1.11	9.25	0.08	335	3	165.09	67.99	1.40
Pesticide											
Herbicide	32.7	0.24	19.23	0.17	22.72	0.20	25.26	0.23	24.98	2.86	0.21
Fungicide	9.5	0.07	0	0	0	0	0	0	2.38	2.38	0.02
Insecticide	44.4	0.33	6.91	0.06	0	0	0	0	12.83	10.65	0.11
Total	13553.25	100	11261.26	100	11262.21	100	11169.72	100	11811.61	580.95	100
Output											
Grain	71747.1	39.68	47101.25	31.21	58371.25	34.47	67987.5	36.34	61301.78	5507.92	35.63
Straw	109072.9	60.32	103820	68.79	110973.3	65.53	119093.75	63.66	110740	3168.93	64.37
Total	180820	100	150921.3	100	169344.6	100	187081.3	100	172041.8	28936.55	100

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By comparing different types of inputs in the four scenario, we see that the percentage of total energy consumed (nitrogen input) with total mean of 4492.14 MJ ha^{-1} (38.03 per cent) is on the top of the chart. The highest amount is for the improved scenario with 49.91 per cent (5572.2 MJ ha^{-1}). Scenario I bags second place with 39.50 per cent (5358.2 MJ ha^{-1}). Other ranks were of scenario II and III with nitrogen inputs of 31.63 and 30.84 per cent (3561.87 and 3473.29 MJ ha^{-1}, respectively). After the energy input of nitrogen, energy input of the fuel (3069.56 MJ ha^{-1} and 25.99 per cent) and seed (3045.75 MJ ha^{-1} and 25.79 per cent) had the highest amount. About the energy of the consumed fuel, scenario III got the first rank with 34.97 per cent of the total input and scenario IV bagged the last rank with 17.82 per cent of the total input. Scenarios I and II with 28.77 and 28.86 per cent of the consumed fuel, got the second and third place. By comparing the four scenarios about the seed consumed, it was seen that the fields of scenario I had the lowest amount with 2820.6 MJ energy (21.81 per cent); scenario III with 2862.58 MJ was placed before it. Scenarios II and IV with the consumption of 3359.8 and 3140 MJ ha^{-1} (29.84 and 28.11 per cent respectively) showed the highest amount of input energy for the seed *(Table 4)*. Findings show that in the region's farming culture, the energy input belonged to the nitrogen chemical fertilizer, fuel and seed. The main reason for this is that wheat production method in the region is traditional. To strong then the field, farmers used great amounts of chemical fertilizers, which utilized in the wrong way without paying attention to organic materials and biological resources.

Other inputs in the fields under study didn't show noticeable measures in different scenarios. The lowest inputs belonged to consuming pesticides. In most fields under study in the four scenarios, farmers did not use insecticides or fungicide. Also, little herbicide was used. The energy input for labor was little considering conversion coefficient. The energy input of machinery with the mean of 511.25 MJ ha^{-1} and 4.33 per cent of the total, got the fourth rank which was higher in scenarios I and III compared to scenarios II and IV. Moreover, the energy input of potassium (K_2O) and phosphorus (P_2O_5) with the means of 462.12 and 165.09 MJ ha^{-1} had 3.91 and 1.4 per cent of the total energy input, respectively *(Table 4)*.

An average of the total production energy in the four scenarios was 172041.8 MJ ha^{-1} and 35.63 per cent of it belonged to grain production energy (61301.78 MJ ha^{-1}) while 64.37 per cent of it belonged to straw energy (110740 MJ ha^{-1}). The highest energy production was obtained in the improved planting methods 187081.3 MJ ha^{-1} and 36.34 per cent of it belonged to grain energy (67987.5 MJ ha^{-1}) while 63.66 per cent of it belonged to straw energy (119093.75 MJ ha^{-1}). Scenario I with 180820 MJ ha^{-1} energy output got the second rank which consisted of 39.68 per cent grains (71747.1 MJ ha^{-1}) and 60.32 per cent straw (109072.9 MJ ha^{-1}). The next fields with scenarios II and III got the next places with 150921.3 and 169344.6 MJ ha^{-1}, respectively *(Table 4)*. The main reason for the observed differences between the amount of input energy and output energy in the four scenarios under study is the difference in the ways to manage things and amounts of input consumption.

The highest energy input coming from fuel in the four production scenarios is about land preparation operation where scenario III showed the highest amount of fuel consumption (1649.77 MJ ha^{-1}) and scenarios I and II with the consumption of 1557.6 and 1428.8 MJ ha^{-1} bagged the second and third places, respectively. By managing the field in the improved scenario, energy of the consumed fuel (452.2 MJ ha^{-1}) decreased significantly. After land preparation operation, the harvest operation had the highest

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amount of fuel input, where scenario III and I (1595.11 and 1313.09 MJ ha^{-1}) were significantly higher than scenarios II and IV (1192.75 and 1187.5 MJ ha^{-1}). Planting operation got the third place with regard to fuel input. It was significantly lower in the improved method than in the other three scenarios. In all four scenarios, the operations of weed control, plant protection, nutrition and transportation got the next places, respectively. Considering that all fields in all four scenarios were dry land planting, no energy input was recorded for irrigation (*Figure 1*).

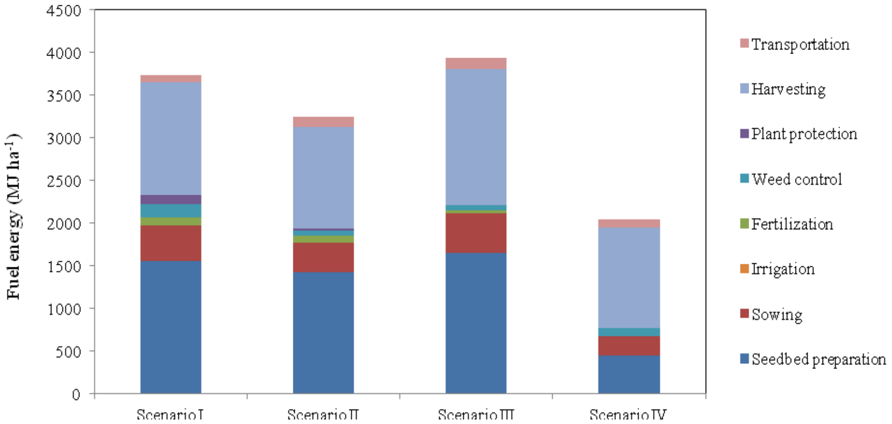

Figure 1. *Energy consumption in fuel in each production operation in each scenario of wheat production. Note that scales are different for the panels*

The energy input of the machinery in different stages of wheat production shows that the biggest part of it in the four scenarios was land preparation which was 284.87, 236.8 and 281.73 MJ ha^{-1} for scenarios I, II and III, respectively. In scenario IV, it was 99.69 MJ ha^{-1}. The operations of planting and harvesting got the next places which were different in different scenarios. Weed control and transportation of the machinery were placed lower. Energy consumption of the machinery for nutrition and protection was little: it was considered zero in the improved scenario. Moreover, because there was no irrigation in the four scenarios, this section did not have an energy input (*Figure 2*).

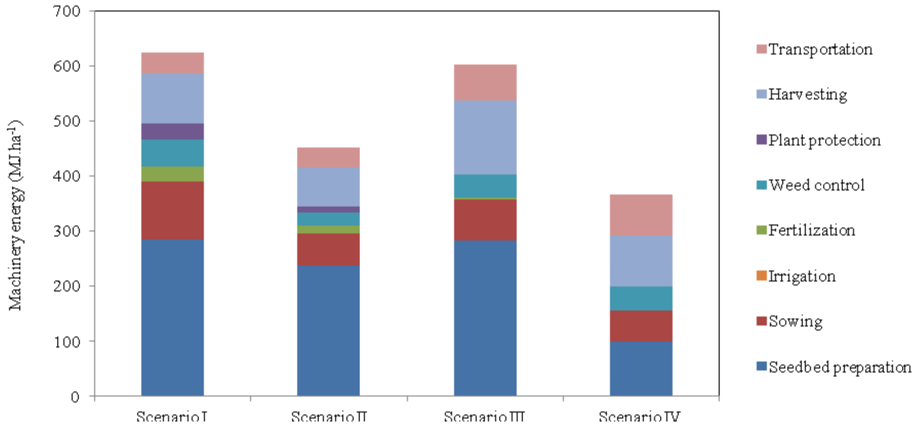

Figure 2. *Energy consumption in machinery in each production operation in each scenario of wheat production. Note that scales are different for the panels*

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Most energy for labor was in the scenarios number I to III for land preparation with 8.9, 7.4 and 8.8 MJ ha^{-1}, respectively. But in the improved scenario, the highest amount of labor energy was nutrition and planting (7.78 and 7.58 MJ ha^{-1}, respectively), and land preparation operation (3.12 MJ ha^{-1}) stood in the next place. Labor energy input was not observed in this scenario for protection. Irrigation in scenarios I and II nutrition bagged the second place and planting operation got the third rank. But in scenario III harvesting and planting operation got the next places (*Figure 3*).

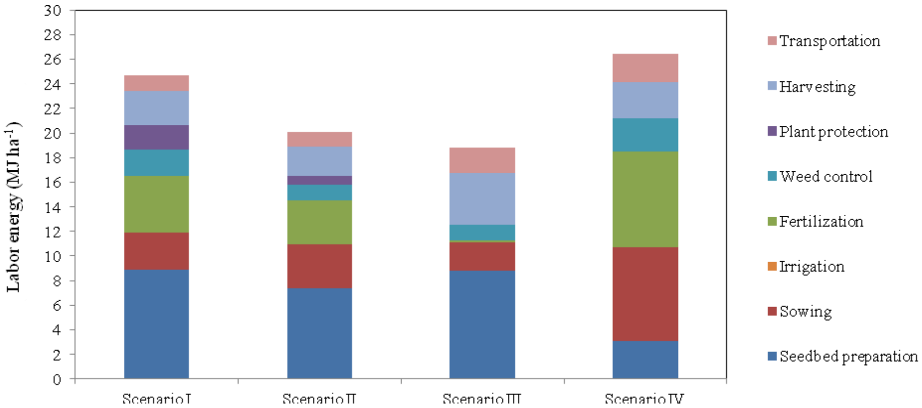

Figure 3. *Energy consumption in labour in each production operation in each scenario of wheat production. Note that scales are different for the panels*

Portions of energy input in wheat production

About different forms of energy, the findings in *Table 5* show that the means for direct and indirect total energy input in the four scenarios were 3295.09 and 8516.52 MJ ha^{-1}, respectively. The highest amount of directly consumed energy was for scenarios III and I with 3968.91 and 3924.35 MJ ha^{-1}. Scenario II with the input of 3270.23 MJ ha^{-1} bagged the third spot and scenario IV with 2016.86 MJ ha^{-1} was at the bottom of the list. Also, the two scenarios I and IV had the highest amounts of indirect energy inputs (9628.9 and 9152.86 MJ ha^{-1}), respectively. The amounts of indirect energy input for scenarios II and III were 7991.03 and 7293.3 MJ ha^{-1}, respectively (*Table 5*). According to the findings in *Table 5*, the means for renewable and non-renewable energy inputs in the four scenarios were 3071.28 and 8740.33 MJ ha^{-1}, respectively. In a group comparison between the two types of renewable and non-renewable energies, it was observed that renewable input energy in scenarios II and IV (3379.89 and 3166.42 MJ ha^{-1}) was more than in scenarios I and III (2845.25 and 2893.55 MJ ha^{-1} of the total consumed energy), respectively.

The lowest non-renewable input energy (7881.37 MJ ha^{-1}) belonged to scenario II and the highest non-renewable input energy (10708 MJ ha^{-1}) belonged to scenario I. Non-renewable energy in scenarios III and IV were 8368.66 and 8003.3 MJ ha^{-1} (*Table 5*).

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Table 5. *Energy types and indices for each wheat production scenario. * Scenario IV is improved planting method. Scenarios I, II, and III are conventional method*

Indices	Scenario				Mean	Standard error
	I	II	III	IV*		
Input						
Direct (MJ ha^{-1})	3924.35	3270.23	3968.91	2016.86	3295.09	455.02
Indirect (MJ ha^{-1})	9628.9	7991.03	7293.3	9152.86	8516.52	533.44
Renewable (MJ ha^{-1})	2845.25	3379.89	2893.55	3166.42	3071.28	124.82
Non-renewable (MJ ha^{-1})	10708	7881.37	8368.66	8003.3	8740.33	664.01
Total input (MJ ha^{-1})	13553.25	11261.26	11262.21	11169.72	11811.61	580.95
Output						
Grain yield (kg ha^{-1})	5796.8	3204.2	3970.8	4625	4399.2	548.92
Straw yield (kg ha^{-1})	11791.7	11187.5	11958.3	12875	11953.13	349.06
Total output (MJ ha^{-1}	180820	150921.3	169344.6	187081.3	172041.8	7940.37
Output/input ratio (MJ ha^{-1})	13.34	13.40	15.04	16.75	14.57	0.81
Specific energy (MJ kg^{-1})	0.43	0.28	0.35	0.41	0.37	0.03
Energy productivity (kg MJ^{-1})	2.34	3.51	2.84	2.42	2.68	0.27
Net energy ratio (MJ ha^{-1})	167266.75	139660.04	158082.39	175911.58	160230.19	7762.96

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Analysis of energy indices in wheat production

As shown in *Table 5,* energy ratio was 14.57. The highest energy ratio (16.75) was of the improved scenario and the lowest amounts (13.34 and 13.3) were in case of scenarios I and II. The reason for the low energy ratio can be attributed to their heavy dependence on inputs and use of more energy for production; these inputs are used without considering environmental issues. About the index of energy productivity, research findings showed that the mean for this index in the four scenarios was 0.37 kg MJ^{-1}, the highest amount of which was obtained in scenarios I and IV as 0.43 and 0.41 kg MJ^{-1}, respectively. As for scenarios II and III, the amounts were 0.28 and 0.35 kg MJ^{-1}, respectively (*Table 5*), which were lower than the results of Khan et al. (2010) in Australia. This is probably because of high energy input in Iranian production systems and the region's farming culture. The mean of the specific energy in the four scenarios was 2.68 MJ kg, where scenario II had the highest amount (3.51 MJ kg). The lowest specific energy belonged to scenario I (2.34 kg MJ^{-1}), and for scenarios III and IV it was 2.84 and 2.42 kg MJ^{-1}, respectively *(Table 5)*. Specific energy is the opposite of energy productivity, so its lower mounts show that less energy has been used for production per every unit of yield *(Table 5)*. About productivity and yield of the four scenarios as a system of energy conversion, research findings showed that the mean for net energy index was 160230.19 MJ per hectare. The highest net energy belonged to the improved scenario (175911.58 MJ per hectare) and scenario I (167266.75 MJ per hectare) got the second rank and scenario II (139660.04 MJ per hectare) stood last. Correct management method in the improved scenario and high amounts of inputs led to this result *(Table 5).*

CO_2 emission and global warming potential

According to the findings in *Table 6*, the mean for the 4 scenarios is 894.07 kg eq-CO_2 ha^{-1}. The lowest amount of global warming potential was for scenario I (710.2 kg eq-CO_2 ha^{-1}) and the highest amount of it was in case of scenario I (equal to 933.59 kg eq-CO_2 ha^{-1}). GWP in scenarios III and IV were 748.66 and 765.78 kg eq-CO_2 ha^{-1}, respectively.

Among different activities, nitrogen energy input got the first place in CO_2 emission and global warming in all scenarios with the average of 327.04 kg eq-CO_2 ha^{-1}, equal to 41.42 per cent. It showed a considerable difference compared to other inputs where the improved scenario stood first with 405.88 kg eq-CO_2 ha^{-1} and scenario I stood second rank with 390.15 kg eq-CO_2 ha^{-1}. Scenarios II and III were in next places with 259.27 and 252.86 kg eq-CO_2 ha^{-1}.

After nitrogen fertilizer, the consumed fuel had the highest CO_2 emission the mean of which in the four scenarios was 254.03 kg eq-CO_2 ha^{-1}, equal to 32.17 per cent, where the improved scenario stood last with 155.25 kg eq-CO_2 ha^{-1}. Scenario III and I got first and second places with 307.17 and 304.17 kg eq-CO_2 ha^{-1} *(Table 6)*. Machinery with average of 63.98 and seed with average of 87.83 kg eq-CO_2 ha^{-1} comprise 8.10 and 11.12 per cent of the total CO_2 emission in the four scenarios. Moreover, global warming potential coming from the use of herbicide had the average of 2.57 kg eq-CO_2 ha^{-1} and 0.33 per cent of the total CO_2 emissions. The portion of other activities in production and transportation of the agricultural inputs was not significant *(Table 6).* The mean for the global warming potential of all scenarios in the unit of area was 894.07 kg eq-CO_2 ha^{-1} which was minimum in scenario II, III and IV (710.2, 748.66 and 765.78 kg eq-CO_2 ha^{-1}) and maximum in scenarios I (933.59 kg eq-CO_2 ha^{-1}). In the

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same respect, the total amounts of global warming potential in the unit of area for potato, canola, barley and peas were 3, 1.7, 1.2, 0.7 and 0.7 t eq-CO_2 ha^{-1} (Tzilivakis et al., 2005 b). Also, global warming potential in the unit of energy input had mean of 66.75 kg eq-CO_2 GJ^{-1} in the four scenarios which was minimum in scenario IV (68.56 kg eq-CO_2 GJ^{-1}) and was maximum in scenario II (63.07 kg eq-CO_2 GJ^{-1}). The mean of global warming potential in the unit of energy output was 4.94 kg eq-CO_2 GJ^{-1} in the four scenarios. The improved scenario had the least global warming potential in unit of energy output width 4.35 kg eq-CO_2 GJ^{-1} and scenario III stood second with 4.73 kg eq-CO_2 GJ^{-1}. Scenarios I and II had the highest global warming potential in the unit of energy output with 5.58 and 5.08 kg eq-CO_2 GJ^{-1} *(Table 7).* Mean of the global warming potential in the unit of grains' weight in the four scenarios was 184.2 kg eq-CO_2 t^{-1}. The lowest global warming potential in the unit of grains' weight was observed in scenarios I and IV where 161.05 and 165.57 kg eq-CO_2 t^{-1} were obtained. The highest global warming potential was in scenario II which was 221.64 kg eq-CO_2 t^{-1}. Less global warming potential was seen in the units of area and weight in scenarios I and II compared to other two scenarios. This can be attributed to less consumption of energy input and also higher production rate in this planting scenario *(Table 7).* The comparison between energy input and the resulting CO_2 emission in this research showed that there was a direct relationship between energy input and global warming potential in wheat production scenarios *(Figure 4)*, in a way that for every MJ increase in energy input in the four scenarios, CO_2 emission increased 75.1 kg per hectare. Since fossil fuel is important factors in GHG emissions, especially CO_2, appropriate agricultural operations have to be used.

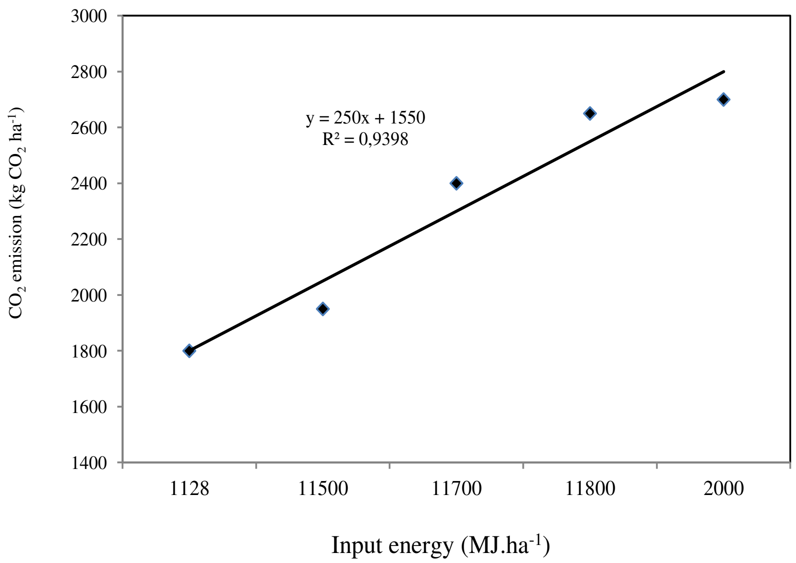

Figure 4. *Liner regression model between input energy and CO_2 emissions in wheat production scenarios*

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Table 6. *CO_2 emissions (kg eq-CO_2 ha^{-1}) for each wheat production scenario. * Scenario IV is improved planting method. Scenarios I, II, and III are conventional method*

Item	Scenario								Mean	Standard error	Share (%)
	I	Share (%)	II	Share (%)	III	Share (%)	IV*	Share (%)			
Seed	91.98	9.85	93.07	13.10	79.30	10.59	86.98	11.36	87.83	2.56	11.12
Machinery	76.91	8.24	57.93	8.16	75.18	10.04	45.89	5.99	63.98	6.03	8.10
Fuel	304.17	32.58	249.54	35.13	307.17	41.03	155.25	20.27	254.03	28.98	32.17
Chemical fertilizer											
N	390.15	41.79	259.27	36.51	252.86	33.77	405.88	53	327.04	33.58	41.42
P_2O_5	46.04	4.93	64/37	5.30	26.83	3.58	42.18	5.51	38.17	3.39	4.84
K_2O	15.42	1.65	10.06	1.42	5	0.67	27	5.53	14.37	3.85	1.82
Pesticide											
Herbicide	3.37	0.36	1.98	0.28	2.34	0.31	2.60	0.34	2.57	0.24	0.33
Fungicide	0.98	0.11	0	0	0	0	0	0	0.25	0.20	0.03
Insecticide	4.58	0.49	0.71	0.1	0	0	0	0	1.32	0.89	0.17
GWP	933.59	100	710.2	100	748.66	100	765.78	100	894.07	40.33	100

Table 7. *Global warming potential equal to kg CO_2 emission per unit area, per unit weight, per unit input and output energy for each wheat production scenario. * Scenario IV is improved planting method. Scenarios I, II, and III are conventional method*

GWP	Scenario				Mean	Standard error
	I	II	III	IV*		
Per unit area (kg eq-CO_2 ha^{-1})	933.59	710.20	748.66	765.78	894.07	40.33
Per unit weight (kg eq-CO_2 t^{-1})	161.05	221.64	188.54	165.57	184.20	13.86
Per unit energy input (kg eq-CO_2 GJ^{-1})	68.88	63.07	66.48	68.56	66.75	1.33
Per unit energy output (kg eq-CO_2 GJ^{-1})	5.58	5.08	4.73	4.35	4.94	0.26

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Discussion

The results showed that non-renewable energies have little portion in the region. This issue is ecologically important because the source of non-renewable energy is generally fossil fuel, and relying on this recourse can bring danger in the future. Research findings show that agriculture in Iran heavily depends on non-renewable energy (Beheshti et al., 2010). High consumption of non-renewable energy decreases the energy productivity of production systems because producing chemicals and using machinery as the main indices of current systems requires the consumption of a lot of energy. In this research the portion of indirect energy is bigger than that of direct energy and the portion of non-renewable energy is higher than that of renewable energy. According to Moore (2010), in order to reach a sustainable production system, we have to increase the energy productivity and portion of renewable energy in the ecosystems. However, nowadays supplying food to the growing population of the world without non-renewable energy seems difficult or perhaps impossible. Therefore, considering the consequences of using chemicals and fossil fuel, agriculture experts will have no other options than think about increasing the sustainability in agriculture and the portion of renewable energy in production systems.

Agriculture is the system of energy conversion; it converts some commercial and non-commercial energy sources into products containing energy which is usable by humans (Kizilaslan, 2009), yield and productivity of this conversion are accessed through indices such as energy ratio, energy productivity and net energy. Energy ratio in Australian and Indian rice planting systems appears to be similar (Khan et al., 2010). According to the results obtained, we can increase energy productivity by decreasing fuel consumption, mechanization and machinery. Moreover, energy consumption decreases when energy resources are used more effectively through optimization of different types of the inputs by choosing the right type, amount, method and time of using the inputs such as chemical pesticides and fertilizers. A research study investigated energy consumption in 97 wheat fields in Marma state, Turkey, and it was observed that wheat production consumed 20653.5 MJ per hectare of energy, where the biggest portion was fuel energy inputs with 45.15 per cent of the total consumed energy and the next was chemical fertilizers with 34.21 per cent (specially nitrogen filled with 31.737 per cent) (Tipi et al., 2009). Other researches also showed that energy ratios in Australian and Indian rice planting systems were similar (Iqbal, 2007; Khan et al., 2010). In another research, the highest amount of fuel consumption and energy input was land preparation operation (Canakci et al., 2005). On the other hand, researches showed that fuel comprises the biggest portion of energy input compared to other direct inputs (Strapatsa et al., 2006). Fuel consumption per unit of field area is affected by factors such as tractor's steam horsepower, depth of plowing, soil type, etc (Kaltsas et al., 2007). Therefore, by analyzing energy input in growing wheat, we realize the use of all energy forms.

The comparison between energy input and global warming potential resulting from them showed that there was a significant relationship between the two. In this regard, Wood and Cowie (2004) stated that CO_2 emission during various agricultural activities either happened directly through consuming fossil fuel or indirectly at the time of production or transportation of the field's needed inputs (herbicides, pesticides and chemical fertilizers). Pathak and Vassmann (2007) stated that agricultural and non-

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agricultural operations (production and transportation of fertilizers and pesticides) in production of rice, made 80-90 and 16-91 kg eq-CO_2 ha^{-1} in global warming potential, respectively. Furthermore, the results of similar researches in olive and sugar beet have shown that consumption of chemical fertilizers, especially nitrogen fertilizer and fossil fuel has had the biggest effect in GHG emission and global warming potential (Kaltsas et al., 2007; Tzilivakis et al., 2005a). In this respect, Soltani et al. (2013) stated that the highest and lowest global warming potentials in the unit of weight were 271.5 and 103.8 kg eq-CO_2 t^{-1}, respectively, and the unit of energy input were 44.6 and 34.8 kg eq-CO_2 GJ^{-1} and in the unit of energy output were 11.7 and 4.5 kg eq-CO_2 GJ^{-1}. In the other research results showed average reduction levels of up to 20% and 25% per material input for spring and summer systems, leading to impact reductions which ranged from 8% to 11% for spring farms and 19% to 25% for summer farms depending on the chosen impact category (Mohammadi et al., 2014). The energy use efficiency was improved about 25% by converting present farms to target units. Furthermore, the GHG emission of each input was investigated for present and optimum units. The results indicated that the total GHG emission of present and optimum farms was calculated as 1847.26 and 1483.52 kgCO_2eq. ha^{-1}, respectively. Moreover, the effect of energy optimization in reduction of GHG emission was found to be as 363.74 kgCO_2eq. ha^{-1} (Nabavi Pelesaraei et al., 2014). Kaltas et al. (2007) investigated the two planting methods organic and conventional in Greece and decided that global warming potential was less in the organic method than in the conventional method. Dayer and Desjardins (2003) investigated the effective management of farm machinery in GHG emission in Canada’s agriculture. They showed that decrease in consumption of fossil fuel reduced GHG emission. The issue of using fossil fuel energy in agriculture is very important because of the preservation of natural resources and also because of the release of greenhouse gases into the atmosphere. Furthermore, developing agricultural systems with the least energy input can help reduce GHG emission in agriculture.

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IRRIGATION AND NITROGEN MANAGEMENT PRACTICES AFFECT GRAIN YIELD AND 2-ACETYL-1-PYRROLINE CONTENT IN AROMATIC RICE

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Abstract. Aromatic rice has a high market value due to its special fragrance and the most important compound in the grains of aromatic rice is 2-acetyl-1-pyrroline (2-AP). In an effort to improve the 2-AP content and the yield of aromatic rice, two known rice cultivars, *Yungao* and *Yundi,* were cultivated across two seasons, and two irrigation and nitrogen management practices were investigated. The results showed that the treatment management practice (TNW) treatment improved panicle number per hill, seed setting rate, and 1000 grain weight and grain yield for both cultivars in early and late season. Significant improvement in grain yield was only observed in *Yundi* in both seasons. Moreover, the 2-AP content in grains was increased for both cultivars in both seasons during grain filling period. Significant increase in 2-AP in grains was observed for *Yungao* at maturity in both seasons, at 7 d AH in early season and at 14 d AH in late season whilst for *Yundi* at 7 d AH, 14 d AH and 21 d AH in early season, and at 14 d AH in late season. Furthermore, 2-AP content in leaves and grains was decrease during grain filling period while proline content in grains was enhanced during grain filling period. Significant relationships were also observed between 2-AP and proline contents in the leaves/grains of both rice cultivars.
Keywords: *aromatic rice; 2-acetyl-1-pyrroline; proline; nitrogen; irrigation*

Introduction

Rice is one of the most important cereal crops and feeds billions of people around the world. Aromatic rice, such as Basmati and Jasmine, has a characteristic fragrance and good grain quality (Ashraf et al., 2017; Ashraf and Tang, 2017). The aromatic rice grains valued at a higher market price than non-aromatic rice varieties (Zhang et al., 2008). Since the 1980s, numerous studies have investigated hundreds of fragrant compounds in scented rice grains and identified the 2-acetyl-1-pyrroline (2-AP) as the key fragrant

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compound of aromatic rice (Yajima et al., 1979; Buttery et al., 1982; Widjaja et al., 1996; Champagne, 2008).

The 2-AP accumulation in aromatic rice is affected by many environmental factors during aromatic rice growth. For example, high night temperature reduced grain quality remarkably (Mohammed and Tarpley, 2010; Nagarajan et al., 2010). During grain filling period, shading treatments significantly improved the 2-AP content and γ-aminobutyric acid (GABA) content in aromatic rice grains (Mo et al., 2015). With salt stress treatment, the popular aromatic rice (*Khao Dawk Mali 105*) cells accumulated Na^+ and proline (Summart et al., 2010) whilst the reduction of fragrant rice yield was related to the fragrance in rice (Fitzgerald et al., 2010). Moreover, some studies reported that salt stress can increase 2-AP content in grains (Gay et al., 2010; Poonlaphdecha et al., 2012). Some minor elements were also found to be associated with 2-AP accumulation in aromatic rice (Lei et al., 2017). Manganese (Mn) application had a positive effect on rice yield, quality and 2-AP content (Li et al. 2016). 2-AP content and proline dehydrogenase (ProDH) activity were increased by moderate concentration of zinc (Zn) and lanthanum (La) treatment (Mo et al., 2016). Furthermore, silicon fertilization also modulates 2-acetyl-1-pyrroline content n fragrant rice (Mo et al., 2017).

Many previous studies have reported that nitrogen and water as important aspects for aromatic rice growth and development. The nitrogen utilization efficiency is associated to the rice genotypes and locations (Djaman et al., 2016). Sikdar et al. (2008) found that N level at 80 kg/hm^2 improved grain quality and soil fertility. However, high nitrogen can lead to lodging in rice (Mahajan et al., 2010). In Asia, rice production is limited due to water shortage (Arora et al., 2006), thus, irrigation patterns directly influence rice production. Alternating wetitnng and drying could regulate rice yield (Zhang et al., 2012), whilst moderate irrigation control can also have a positive impact on 2-AP content and grain yield of aromatic rice (Yoshihashi et al., 2002; Tian et al., 2014). Hakoomat et al. (2014) found the N and Zn interactions had the most significant improvement on yield and yield related traits when the N level was 12kg/hm^2 and Zn level was 14kg/hm^2. Moreover, moderate irrigation at tillering, booting, and grain filling stage could increase the 2-AP content in grains (Tian et al., 2010; Wang et al., 2013a, b). Yang et al. (2012) reported high aroma content in grains was associated to high total nitrogen in soil. Zhong and Tang (2014) found that 2-AP content in grains was increased with increasing nitrogen application. Li et al. (2014) found the 2-AP content in brown rice was highest when the N supply was 60kg/hm^2 and the irrigation potential was -20 kPa at tillering stage.

In our previous studies, we have investigated the interaction effect of water and nitrogen at tillering stage (Li et al., 2014), booting stage and grain filling stage on grain yield and 2-AP accumulation in grains (data unpublished), then, we selected the best water and nitrogen treatment of the three stages and combined the treatments together for investigating the additive effect of the nitrogen and water management treatment on aromatic rice yield and 2-AP accumulation. In this study, we aimed to identify the affects of the combinations of water and nitrogen applications implemented at tillering stage, booting stage, and grain filling stage on 2-AP accumulation and grain yield.

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Materials and Methods

Plant materials and growing condition

Two popular aromatic rice cultivars, *Yungao* and *Yundi*, having 115 -120 days of growth period were planted in early season and late season in 2015 at the College of Agriculture, South China Agricultural University, Guangzhou, China (23°20’ N, 113°30’ E and 11 m above the sea level. The experimental site has a subtropical-monsoon type climate with mean annual air temperatures of 22.4 ºC and mean annual precipitation of 2638.3 mm (Fig. 1). The seeds were soaked in water for 24 h, germinated in manual climatic box for the next 24h, and raised at the Research Farm of the College Agriculture. 30-day-old seedlings were transplanted to the field at the recommended planting distance (30 cm × 12 cm). The experimetal soil was sandy loam with of 25.65% organic matter content, 1.362% total N, 0.958% total P, and 17.520% total K.

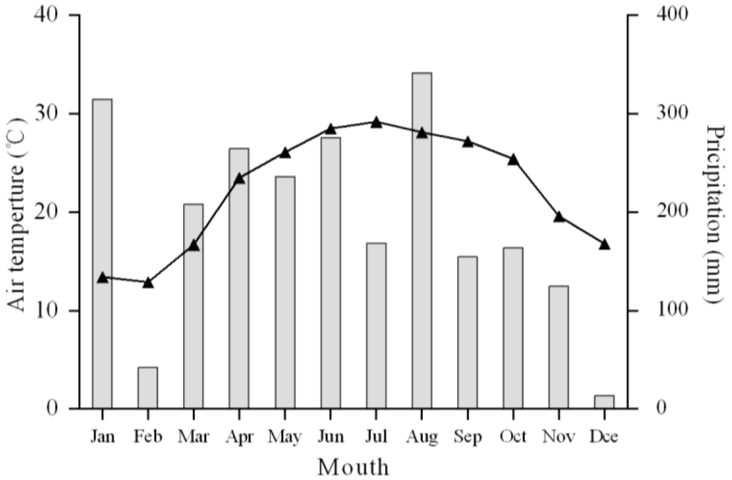

Figure 1. *Mean monthly temperature and precipitation in 2016 of the experimental site*

Treatments and plant sampling

The experiment was conducted in early season and repeated in late season. Experimental treatments are as follows: (i) The conventional management practice (CK) involves applying nitrogen (30 kg/hm^2) at tillering stage, booting stage, and grain filling stage; the water flow at these three stages was 0 kPa in both early and late season rice. (ii) The treatment management practice involved applying nitrogen (60 kg/hm^2) at tillering stage with heavy drought conditions (water potential of - (25±5) kPa), applying 60 kg/hm^2 nitrogen at the booting stage with feebly arid conditions (water potential of - (15±5) kPa), and applying no nitrogen at the grain filling stage with shallow-watered irrigation (water flow of 0 kPa) (TNW). P_2O_5 (90 kg/hm^2), K_2O (195 kg/hm^2) were applied as basic fertilizer. The fresh leaves and grains were sampled from the rice at the end of tillering stage; after 7, 14, and 21 d heading stage; and at maturity. Samples were immediately stored at -20°C for 2-AP analysis.

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Yield and yield related traits

At maturity, the grains were harvested from one unit sampling area (8.1 m^2) in each plot and threshed by machine. The harvested grains were sundried, and the dry weight was used to estimate the grain yield. Thirty hills of rice plants from different locations in each plot were sampled for calculating the average effective panicles number per hill. Then, three hills representative plants were taken for determination of the yield related traits.

Proline estimation

The proline content in leaves and grains were measured according to the method established by Bates et al. (1973). Leaves or grains in which the weight was almost 0.3 g, were homogenized in a 4 ml solution of 3% sulfosalicylic acid and cooled after bringing to a boil for 10 min. Samples were filtered and 2 ml of the filtrate was mixed with 3 mL ninhydrin reagent (2.5 g ninhydrin in 60 mL glacial acetic acid and 40 mL 6 M phosphoric acid) and 2 mL glacial acetic acid. For the extraction of proline, the mixture was boiled for 30 min and 4 mL toluene was added to the cooled liquid. The extract was centrifuged at 4000rpm for 5 min, and proline absorbance was detected at 520 nm. The proline concentration, expressed as $\mu g \cdot g^{-1}$, was assayed by using standard curve to calibrate.

2-AP estimation

The 2-AP content was estimated using the method described by Huang et al. (2012), Prior to analysis, leaves were cut into sections and grains were ground by mortar and pestle. Approximately 5 g leaves or 10 g grains were mixed homogeneously with 150 mL purified water into a 500 mL round-bottom flask attached to a continuous steam distillation extraction head. The mixture was boiled at 150°C in an oil pot. A 30 mL aliquot of dichloromethane was used as the extraction solvent and was added to a 500 mL round-bottom flask attached the other head of the continuous steam distillation apparatus, and this flask was boiled in a water pot at 53°C. The continuous steam distillation extraction was linked with a cold water circulation machine in order to keep temperature at 10°C. After approximately 35 min, the extraction was complete. Anhydrous sodium sulfite was added to the extract to absorb the water. The dried extract was filtered by organic needle filter and analyzed for 2-AP content by GCMS-QP 2010 Plus. High purity helium gas was used as the carrier gas at flow rate of 2 mL/min. The temperature gradient of the GC oven was as follows: 40°C (1 min), increased at $2°C \cdot min^{-1}$ to 65°C and held at 65°C for 1 min, and then increased to 220°C at $10°C \cdot min^{-1}$, and held at 220°C for 10 min. The retention time of 2-AP was confirmed at 7.5 min. Each sample had three replicates, and 2-AP was expressed as $\mu g \cdot kg^{-1}$.

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Treatment design and statistical analysis

This study was arranged as a randomized complete block design (RCBD) with three replicates (n=3). Data were analyzed by SPSS 16.0 software (SPSS Inc., Chicago, USA), and the probability level was set at 5% ($P < 0.05$).

Results

Grain yield and yield related traits

As shown in *Table 1*, the panicle number per hill, seed-setting rate, and 1000-grain weight and yield were higher in TNW treatment than in CK for *Yungao* and *Yundi* in both seasons. The seed setting rate was significantly improved by 11.75% in TNW treatment for *Yundi* in late season. The grain yield was dramatically increased by 12.13% and 21.00% for *Yundi* in early and late season, respectively. CK treatment had higher grain number per panicle than TNW treatment in *Yungao* and *Yundi* in both seasons.

Table 1. *Effects of irrigation and nitrogen management practices on yield and yield related traits in aromatic rice*

Season	Cultivar	Treatment	Panicle number per hill	Grains number per panicle	Seed-setting rate (%)	1000-grain weight (g)	Yield (t hm^{-2})
Early season	Yungao	CK	14.03±0.62a	82.52±2.07a	87.24±0.81a	25.13±0.08a	5.16±0.45a
		TNW	15.48±0.71a	74.92±1.50a	88.58±2.37a	26.20±0.51a	5.74±0.38a
	Yundi	CK	13.05±0.48a	108.80±8.96a	88.60±1.70a	24.68±0.12a	5.36±0.20b
		TNW	13.30±0.55a	93.68±4.00a	90.25±0.96a	24.85±0.30a	6.01±0.07a
Late season	Yungao	CK	12.76±0.49a	111.00±5.41a	81.98±4.72a	25.88±0.22a	4.59±0.27a
		TNW	13.00±0.57a	108.49±4.12a	86.10±0.78a	26.50±0.44a	4.73±0.14a
	Yundi	CK	12.69±0.47a	114.82±3.40a	79.97±2.33b	24.03±0.13a	4.81±0.08b
		TNW	12.77±0.47a	109.65±10.79a	89.37±2.34a	24.60±0.44a	5.82±0.07a

Means in the same column followed by different lower case letters for the same variety differ significantly at $P < 0.05$ by T-test. CK (control); TNW (60 kg/hm^2 nitrogen and water potential of - (25±5) kPa) were applied at the tilling stage, 60 kg/hm^2 nitrogen and water potential of - (15±5) kPa were applied at the booting stage, no nitrogen and water flow of 0 kPa were applied at the grain filling stage)

2-AP content in grains

We observed higher 2-AP content in grains in TNW treatment than CK for both cultivars at all the sampling stages in the early and late season. For *Yungao*, the 2-AP content in grains were significantly increased by 61.88% and 31.10% at 7 d AH and maturity in early season, respectively; significant increase in 2-AP content in grains by

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79.02% and 13.88% were observed at 14 d AH and maturity in late season, respectively. For *Yundi*, the 2-AP content in grains in TNW treatment were remarkably higher than CK at 7 d AH, 14 d AH and 21 d AH in early season; significant improved in 2-AP content in grains by 12.07% was found at 14 d AH in late season.

Table 2. *Effects of irrigation and nitrogen management practices on 2-AP content of grains at different stages (μg·kg^{-1})*

Season	Cultivar	Treatment	7 d AH	14 d AH	21 d AH	Maturity
Early season	Yungao	CK	102.01±0.46b	115.56±5.75a	145.84±8.09a	387.30±24.94b
		TNW	165.13±6.80a	129.26±5.21a	146.11±3.41a	507.76±27.14a
	Yundi	CK	119.68±2.35b	67.55±1.01b	94.46±5.75b	104.42±11.50a
		TNW	176.14±1.82a	124.14±3.96a	119.07±2.21a	120.91±2.88a
Late season	Yungao	CK	139.24±0.22a	126.77±1.12b	140.77±3.17a	255.99±3.01b
		TNW	140.65±1.22a	226.94±5.48a	146.54±0.07a	291.51±8.48a
	Yundi	CK	146.43±0.85a	233.22±4.00b	164.51±7.47a	327.59±1.44a
		TNW	164.93±5.17a	261.36±5.93a	176.73±6.73a	336.91±3.11a

Means in the same column followed by different lower case letters for the same variety differ significantly at P <0.05 by T-test. AH=after heading; CK (control); TNW (60 kg/hm^2 nitrogen and water potential of - (25±5) kPa) were applied at the tilling stage, 60 kg/hm^2 nitrogen and water potential of - (15±5) kPa were applied at the booting stage, no nitrogen and water flow of 0 kPa were applied at the grain filling stage)

2-AP content in leaves

Table 3 shows that TNW treatment decrease 2-AP content in leaves at all the sampling stages. In early season, 2-AP content in leaves were significantly decreased except for 21 d AH and 14 d AH for *Yungao* and *Yundi*, respectively. The 2-AP content in leaves in TNW treatment decreased for both rice cultivars at 14 d AH, and for *Yungao* at the maturity.

Table 3. *Effects of irrigation and nitrogen management practices on 2-AP content of leaves at different stages (μg·kg^{-1})*

Season	Cultivar	Treatment	7 d AH	14 d AH	21 d AH	Maturity
Early season	Yungao	CK	401.96±4.12a	245.84±16.62a	109.84±2.61a	418.81±7.14a
		TNW	342.24±11.31b	121.43±4.21b	109.51±0.16a	284.18±0.58b
	Yundi	CK	356.64±6.33a	212.29±7.24a	221.28±6.60a	217.18±5.18a
		TNW	112.16±6.57b	211.61±3.73a	117.84±6.60b	112.13±3.30b
Late season	Yungao	CK	347.59±5.19a	200.41±6.73a	238.54±4.61a	238.57±10.19a
		TNW	239.16±11.31b	190.39±2.90a	217.04±6.33a	199.82±2.70b
	Yundi	CK	343.59±2.56a	170.41±4.97a	209.46±12.09a	198.72±15.5a
		TNW	283.23±11.49b	167.32±5.78a	199.58±0.85a	197.52±5.77a

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Means in the same column followed by different lower case letters for the same variety differ significantly at P <0.05 by T-test. AH=after heading; CK (control); TNW (60 kg/hm^2 nitrogen and water potential of - (25±5) kPa) were applied at the tilling stage, 60 kg/hm^2 nitrogen and water potential of - (15±5) kPa were applied at the booting stage, no nitrogen and water flow of 0 kPa were applied at the grain filling stage)

Proline content in grains

The proline content in grains increased at all the sampling stages during grain filling in the early and late season. For *Yungao*, the proline content significantly enhanced at 21 d AH and maturity in early season and remarkably increased at 7 d AH, 14 d AH and 21 d AH in the late season. For *Yundi*, TNW treatment significantly increased proline content in grains at 7 d AH in early season, significantly improved proline content in grains at 7 d AH and 14 d AH in late season was observed (*Table 4*).

Table 4. *Effects of irrigation and nitrogen management practices on proline content on grains at different stages (μg·g−1)*

Season	Cultivar	Treatment	7 d AH	14 d AH	21 d AH	Maturity
Early season	Yungao	CK	41.17±3.72a	46.82±2.44a	28.11±0.17b	25.31±0.04b
		TNW	48.89±0.39a	47.45±3.13a	36.77±0.27a	26.53±0.05a
	Yundi	CK	20.60±0.46b	14.69±0.98a	22.58±0.07a	22.52±0.03a
		TNW	45.66±1.73a	20.91±4.30a	22.71±1.21a	23.64±0.77a
Late season	Yungao	CK	43.23±1.80b	61.02±3.10b	43.84±0.85b	7.36±0.80a
		TNW	52.97±1.97a	79.79±1.53a	60.42±1.37a	9.50±0.79a
	Yundi	CK	44.70±6.39b	42.22±3.03b	30.41±4.00a	5.26±1.56a
		TNW	48.84±2.57a	56.09±2.30a	35.51±0.98a	8.13±1.76a

Means in the same column followed by different lower case letters for the same variety differ significantly at P <0.05 by T-test. AH=after heading; CK (control); TNW (60 kg/hm^2 nitrogen and water potential of - (25±5) kPa) were applied at the tilling stage, 60 kg/hm^2 nitrogen and water potential of - (15±5) kPa were applied at the booting stage, no nitrogen and water flow of 0 kPa were applied at the grain filling stage).

Proline content in leaves

We observed a decrease in proline content in leaves at all the sampling stages in the early and late season. For *Yungao*, significant decreased in proline in leaves was found at the 7 d AH and maturity in the early season; a significant decrease in proline in leaves was detected at 7 d AH, 14 d AH and 21 d AH in late season. For *Yundi*, TNW treatment showed significantly lower in proline content in leaves than CK at 7 d AH, 21 d AH and Maturity in early season, and at 7 d AH and 14 d AH in late season (*Table 5*).

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***Table 5.** Effects of irrigation and nitrogen management practices on proline content in leaves at different stages (μg·g^{-1})*

Season	Cultivar	Treatment	7 d AH	14 d AH	21 d AH	Maturity
Early season	Yungao	CK	99.37±4.04a	52.14±3.59a	15.31±1.32a	22.08±0.82a
		TNW	86.78±0.40b	45.99±2.77a	14.33±0.09a	17.62±0.81b
	Yundi	CK	94.55±1.00a	67.94±0.32a	20.72±0.03a	20.75±0.01a
		TNW	59.64±1.76b	54.27±5.20a	12.54±0.80b	11.82±0.36b
Late season	Yungao	CK	20.53±0.72a	27.79±0.35a	17.66±0.97a	12.30±0.55a
		TNW	17.77±0.30b	9.29±0.46b	13.63±0.15b	8.69±1.40a
	Yundi	CK	23.11±1.08a	15.86±0.03a	16.05±0.73a	16.75±0.06a
		TNW	17.01±1.18b	14.67±0.14b	15.09±0.34a	15.05±1.47a

Means in the same column followed by different lower case letters for the same variety differ significantly at P <0.05 by T-test. AH=after heading; CK (control); TNW (60 kg/hm^2 nitrogen and water potential of - (25±5) kPa) were applied at the tilling stage; 60 kg/hm^2 nitrogen and water potential of - (15±5) kPa were applied at the booting stage; no nitrogen and water flow of 0 kPa were applied at the grain filling stage).

Correlation analysis

There existed a significant positive correlation between yield and seed-setting rate (r=0.8402, P<0.01) and a significant negative correlation between effective panicles and grain number per panicle (r=-0.9335, P<0.01) was observed.

***Table 6.** Relationship between yield and yield related traits of two rice cultivars*

Paramaters	Panicle number per hill	Grains number per panicle	Seed-setting rate	1000-grain weight
Grains number per panicle	-0.9335**			
Seed-setting rate	0.3906	-0.5014		
1000-grain weight	0.4348	-0.3419	0.0870	
Yield	0.3979	-0.4836	0.8402**	-0.2389

Significant correlations at *P < 0.05 and **P < 0.01. AH= after heading; MS= maturity stage.

The 2-AP content in grains at maturity was significantly positive correlated to the 2-AP content in grains at 21 d AH (P <0.01), the 2-AP content in grains at 14 d AH (P <0.01 for early season, P <0.05 for late season), and the proline content in grains at 7 d AH in early season (P <0.05). However, the 2-AP content in grain at maturity showed significant negative correlation with the proline content in leaves at 14 d AH (P <0.05),

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the proline content in leaves at 21 d AH (P <0.05 in early season, P <0.01 in late season)(*Fig. 2 a-e*).

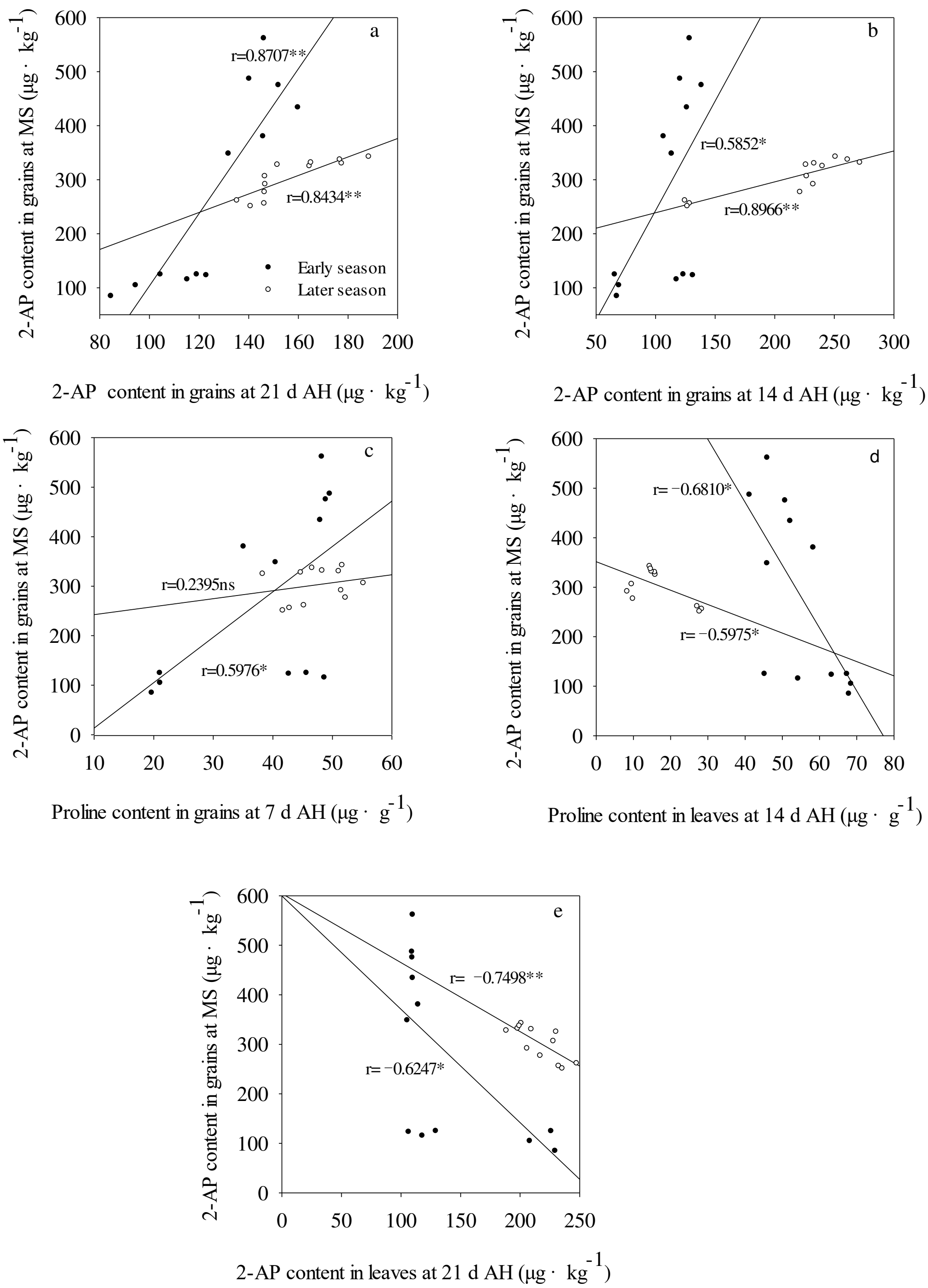

Figure 2. *Correlation analyses between the 2-AP content in grains at maturity and (a) the 2-AP content in grains at 21 d AH, (b) the 2-AP content in grains at 14 d AH, (c) the proline content in grains at 7 d AH, (d) the proline content in leaves at 14d AH, (e) the 2-AP content in leaves at 21 d AH. Significant correlations at *P < 0.05 and **P < 0.01. AH= after heading; MS= maturity stage*

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The 2-AP content in leaves at 21 d AH showed significant positive correlation with the proline content in leaves at 14 d AH and 21 d AH (P <0.01 in early season, P <0.05 in later season) (*Figure 3 a-b*).

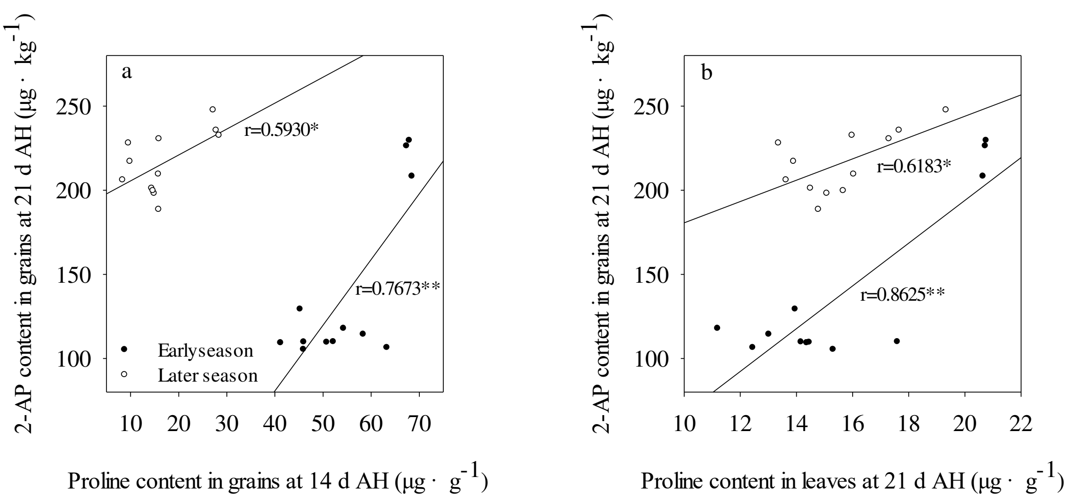

Figure 3. *Correlation analyses between the 2-AP content in leaves and proline content in leaves at 14 d AH and 21 d AH. Significant correlations at *P < 0.05 and **P < 0.01. AH= after heading; MS= maturity stage*

Discussion

The biosynthesis and accumulation of 2-AP in aromatic rice is an important phenomenon that is affected by several factors (Mo et al., 2016). Precious studies have reported some precursors in the pathways of 2-AP biosynthesis, for example, Seitz et al. (1993) and Huang et al. (2012) found proline was the most important precursor and directly involved in 2-AP formation, then, ornithine and glutamic acid were found to have relevant relationship with the fragrance constitution (Yoshihashi, 2002). The fragrance of aromatic rice is affected by environmental factors, in particular, the irrigation model and nitrogen application. For water irrigation, Wang et al. (2013b) found that moderate soil water potential (- (25±5) kPa) at the tillering stage significantly enhanced in 2-AP accumulation in grain by 22.70%-29.76%. Similar findings were also observed when the soil water potential was - (25±5) kPa at the booting stage, the 2-AP content in grain was significantly increased by 35.88%-47.02% (Wang et al., 2013a). Moreover, when the shallow-water irrigation method was applied at grain filling stage, the 2-AP content in grains was markedly increased by 54.80%-322.90% (Tian et al., 2010). The enhancement in 2-AP content in grains was associated to the improvement in proline content in grains (Tian et al., 2010; Wang et al., 2013a,b). Moreover, nitrogen application also has positive influence on the accumulation of proline and 2-AP. Yang et al. (2012) reported higher proline was found in aromatic rice grains which was planted in paddy filed contains higher total nitrogen, and higher proline content contributed to the accumulation of higher aroma.

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Li et al. (2014) found that 2-AP content in grains increased significantly by 10.33%-23.50% when 60 kg/hm^2 nitrogen fertilizer was applied at the tillering stage. Zhong and Tang (2014) also found that the grains 2-AP content was increased, because of the proline concentrations in leaves and grains were improved with the increasing nitrogen application. Meanwhile, the effects of water deficit and nitrogen interaction on proline content have been reported in some previous studies. Lalelou and Fateh (2014) reported that under severe water conditions (60% field capacity loss) and 80 kg/hm^2 nitrogen, the proline content in flag leaf of wheat was increased (15.76-20.39 fold). All the previous indicated the single water or nitrogen treatment could improve the gain 2-AP content. According our previous studies (data unpublished), we have found the water-nitrogen treatment improved the grain 2-AP content at tillering stage, booting stage, and grain filling stage. In this study, we have further found that 2-AP content in grains was increased by TNW (The treatment management practice involved applying nitrogen (60 kg/hm^2) at tillering stage with heavy drought conditions (water potential of - (25±5) kPa), applying 60 kg/hm^2 nitrogen at the booting stage with feebly arid conditions (water potential of - (15±5) kPa), and applying no nitrogen at the grain filling stage with shallow-watered irrigation (water flow of 0 kPa)) (*Table 2*). Similarly, the proline concentration in grains at the four sampling stages was increased in early and later seasons (*Table 3*). Moreover, the 2-AP content in grains at maturity was significantly positive correlated to the 2-AP content in grains at 21 d AH, the 2-AP content in grains at 14 d AH, and positive correlation between 2-AP in grain at maturity and proline content in grain at 7 d AH was also observed (*Fig. 2 a-c, e*). This indicated that the TNW treatment can increase 2-AP content in grains at maturity due to the improvement of 2-AP content in grains at 14 d AH and 21 d AH and the proline content in grains at 7 d AH.

Interestingly, we observed that the proline content and 2-AP content in leaves at the four sampling stages was decreased (*Table 4, 5*). Correlation analysis indicated that the 2-AP content in grains at maturity showed significant negative correlation with the proline content in leaves at 14 d AH and the proline content in leaves at 21 d AH (*Fig. 2d*). Further, the 2-AP content in leaves at 21 d AH showed significant positive correlation with the proline content in leaves leaf at 14 d AH and 21 d AH (*Fig. 3 a-b*). This suggested that proline or 2-AP may possibly transport from leaves to grains (Buttery et al., 1983; Maraval et al., 2010; Mo et al., 2016).

The water and nitrogen effects on crop yield have been investigated by many researchers (Wang et al., 2003; Cai et al., 2006; Yang et al., 2016). Wang et al. (2016) found that 200 kg/hm^2 nitrogen fertilizer application and alternate wetting and moderate drying regime increased grain yield due to more panicle and more spikelet number of per panicle; 300 kg/hm^2 nitrogen fertilizer application and alternate wetting and serve drying regime treatment increased the number of panicle, spikelet number per panicle and the percentage of filled grains and produced high yield. Li et al. (2014) found that the yield aromatic rice varieties were increased under the interaction of 60 kg/hm^2 nitrogen fertilizer application and water flow of - 10 kPa because of higher seed-setting rate, 1000-grains weight or number of panicles. Our results also confirmed that water and nitrogen applications have a positive effect on the yield of two fragrant rice cultivars. The

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improvement in grain yield is associated to the higher panicle number per hill, seed-setting rate, and 1000 grain weight. Moreover, we found significant positive correlation between grain yield and seed-setting rate (r=0.8402, P <0.01) (*Table 1, 6*).

Conclusion

In conclusion, the TNW treatment increased grain yield, and some yield parameters, 2-AP and proline in grains, and led to improve the grain aroma. For revealing the mechanism of 2-AP transportation by irrigation and nitrogen management practice treatment, much work should be done at molecular and physiological level.

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THE REMOVAL OF LEAD (II) IONS FROM AQUEOUS SOLUTIONS BY ACID-ACTIVATED CLAY MODIFIED WITH SODIUM CARBOXYMETHYL CELLULOSE

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Abstract. The suitability of clay modified with sodium carboxymethyl cellulose was tested for the removal of Pb^{2+} from aqueous solutions. The effect of pH on the adsorption process was investigated in the pH range from 1 to 9. Tests were conducted at different temperatures: 20 °C, 30 °C, 40 °C, 50 °C and 60 °C. The effect of initial concentration of adsorbate on lead removal was analysed using solutions with lead concentrations from 50-150 mg L^{-1}. Also, the amount of adsorbent in the process of removing lead was determined. The efficiency of lead removal was observed to be 86.19 % from solution with the initial concentration 100 mg L^{-1}. Obtained Freundlich adsorption isotherms show extremely high correlation coefficient R^2, i.e. processes of equilibria on the investigated clays can be described using this equation. The series of conducted experiments shows that the clay modified with carboxymethyl cellulose is suitable for the removal of lead from waste waters. Further researches should be focused on modifying clay with other long chain molecules which can improve adsorption properties.
Keywords: *adsorption, ICP-OES, scanning electron microscopy, FTIR spectral analysis, waste waters*

Introduction and literature review

Heavy metals are very toxic and that their discharges from industrial plants into waste waters cause harmful effects on human health and the environment is well known. Due to the lead production and its widespread use, it can contaminate air, soil, and via them enter the food chain through food of plant and animal origin. Main sources of lead in the environment are petrol to whom tetraethyl lead is added as a detonator (in many countries lead is fallen into disuse with this purpose (WHO, 2011) metal industry, industrial waste, paint colours, lead water pipes in old buildings etc) (Mirić and Šorbajić, 2002). According to the data of World Health Organization (2011), concentration of lead in water should be below 10 μg L^{-1}.

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Lead is one of the oldest known toxicants. Around 10 % of lead is getting absorbed from digestive tract. According to IARC (International Agency for Research on Cancer), lead is belonging to group 2 of the potential human carcinogen agent (Mirić and Šorbajić, 2002). Exposure to lead during pregnancy affects the DNA methylation status of the fetal germ cells, which leads to altered DNA methylation in grandchildren's neonatal dried blood spots (Sen et al., 2015). Long exposure to low doses mainly harms nervous system (these changes occur at concentrations in blood from 100-150 μg L^{-1}). Mechanism of its toxicity on nervous system can be one of the following: direct neurotoxicity (apoptosis excitotoxicity, influences on neurotransmitter storage and release process), calcium replacement (lead has ability of passing through blood-brain barrier; back-transport of lead via the Ca-ATPase pump) (Lidsky and Schneider, 2003). Toxic effects of lead are manifested as renal failure, hypertension, fertility problems and spontaneous miscarriage, and even fatal outcome due to problems with cardiovascular system (WHO, 2011). Toxic effects are consequences of the inactivation of particular enzyme systems, binding to SH groups of proteins, replacements of essential elements (Mirić and Šorbajić, 2002). Some techniques for removing lead from water are: removing by coffee grounds as vegetable biomass (Tokimoto et al., 2004); adsorption onto calcined phosphate (Aklil et al., 2004); adsorption onto zeolites (Scott et al., 2002; Panayotova and Velikov, 2002), adsorption onto clays (Celis et al., 2000; Cooper et al., 2002). Addition of polymers on the physico-chemical properties of bentonite was tested for cleaning water solutions (Stojiljkovic et al., 2014). All mentioned techniques are relatively cheap. Therefore, it is an imperative to find suitable and cheap technique for the removal of toxic metal from the environment.

Generally, techniques developed for the removal of toxic metals from water solutions include precipitation, ion exchange, adsorption, filtration, electrodeposition, reversed osmosis (Rao et al., 2010). Clay is finely granulated soil, which includes combination of one or more clay minerals and metal oxides or organic substances contained in trace amounts (Guggenheim and Martin, 1995). Adsorption of heavy metals was investigated in several works using the clay as the adsorbent: lead on the bentonite (Zhu et al., 2008), lead on the clay activated with acid (Resmi et al., 2012), chromium on the modified clay (Arfaouia et al., 2008), and Inglezakisa et al. (2007) achieved the efficiency of 100 % in the removal of Pb^{2+} from water solutions using clinoptilolite and bentonite as adsorbents. The influence of different factors affecting the adsorption was investigated, such as pH, agitation time, temperature, quantity of adsorbents, concentration of adsorbate, kinetics of adsorption (Resmi et al., 2012). Maximal adsorption capacity of natural clays for Pb^{2+} ion is 104.28 mg g^{-1} (Zhu et al., 2008). The efficiency of the removal of lead ions using acid activated clay is equal to 92.4 % at the initial concentration 100 mg L^{-1} at pH=6 and 30 ^{0}C (Resmi et al., 2012). Bentonite is used in food industry as an anticaking agent, helping prevent food particles from sticking together (E 558). There are no data in the literature that anyone performed the removal of lead from aqueous solutions using the bentonite modified with sodium carboxymethyl cellulose (NaCMC), a food additive labeled as E 466 (Mirić and Šorbajić, 2002).

In this paper it was investigated the efficacy of the clay modified with NaCMC for the removal of lead from aqueous solution by adsorption and monitoring of the influence of pH value, temperature, dose of the adsorbent and the concentration of adsorbate was performed. The aim of this work was to create a new adsorption material,

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clay modified with long chain molecule (in this case carboxymethil cellulose), with improved properties for heavy metal ions adsorption (Zhu et al., 2008). New adsorption material provides simple and cheap technique for removing Pb(II) ions from waste waters.

Material and methods

Reagents

NaCMC was purchased from Weifang Lude Chemical Co., Shandong China. For the acid activation of clay (bentonite-Riznica prirode, Serbia) concentrated HCl (p.a. Zorka Pharma, Serbia) was used. Lead nitrate (p.a. Alkaloid Skopje) was used for the preparation of adsorbate solution. Multistandard-UltraScientific Analytical Solutions (USA) were used for the quantitative determination of lead. Deionized water was used for the preparation of all solutions.

Apparatus

The following apparatus were used: ICP-OES iCAP 6000 (Thermo Scientific, United Kingdom), FTIR spectrometer Bomem MB-100 (Hartmann & Brown, Canada) with standard DTGS/KBr detector, scanning electronic microscope JSM-5300 (JEOL, Japan), the device for the preparation of samples for electron microscopy JFC-1100E ION SPUTTER (Jeol Co., Japan), digital pH meter (HANNA, USA), and hothouse (Trade Raura, Spain).

Adsorbent

Acid activated clay was prepared by the treatment of crude clay with concentrated HCl. 15 g bentonite clay was suspended in the beaker with 300 mL deionized water. Afterwards NACMC was added in the quantity of 1 % of clay mass (0.15 g) (modified clay GI), 3 % (GII) and 5 % (GIII) and then heated at 90 °C and mixed on rotary mixer (1-2 h). 5 mL of conc HCl was added dropwise. The probe was left overnight, and then filtered through Buchner funnel on vacuum pump and rinsed with plenty of deionized water until negative reaction on chlorides. Modified clay was dried in the oven at 110 °C to constant weight, left overnight and then smashed in the mortar. Before the addition of NaCMC pH was 9.2±0.1, while pH after the addition of NaCMC and HCl was 1.35±0.1.

FTIR spectral analysis

Samples of carboxymethyl cellulose, modified clays (GI, GII, GIII) and unmodified clay (G0) were recorded on FTIR spectrometer. Scanning was performed in the range of wavelengths from 4000-400 cm^{-1} with the resolution 4 cm^{-1}.

SEM analysis

SEM analysis of surface samples was performed by scanning electron microscope JEOL JSM-5300, which is operated at a working potential of 30 kV, and the depth of penetration of electron beam was 10 pm. Characteristics of surface samples are observed at different magnifications (1000×, 2000×, 5000×). SEM-EDS analysis was performed using the same scanning microscope, but with the detector (probe) Linx Analytical QX 2000b.

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Adsorbate

The stock solution of Pb^{2+}ions (1000 mg L^{-1}) were prepared by weighing exactly known mass of $Pb(NO_3)_2$ and dissolving in deionized water in volumetric flask. Thus solution was used for further investigations by diluting to required concentrations.

Adsorption study

Particular mass of adsorbent was added into the beaker with 50 mL of lead nitrate solution of particular concentration. Solutions were mixed for 20 min on the rotary mixer (speed 150 rpm) at particular temperature, and filtered through filter paper (Whatman Grade 589). Concentration of the lead in the filtrate was determined using optical emission spectrometry with inductively coupled plasma (ICP-OES) (Yi et al., 2011). The influence of the temperature was investigated by heating on the water bath, and the influence of pH by the addition of HCl and NaOH solutions to solution of Pb^{2+} ions (Adhikari and Singh, 2003).

Adsorption capacity can be calculated according to the equation (Eq. 1):

$$q_e = (C_o - C)\frac{V}{m} \qquad \text{(Eq. 1)}$$

where c_0 is the initial concentration of Pb^{2+} ions, c remaining concentration of Pb^{2+} in the state of equilibrium, V volume of the solution and m mass of the adsorbent (Yasemin and Zeki, 2007).

Results and discussion

Characterization of adsorbents

Scanning electron microscopy

According to the producer specification, bentonite clay contains minerals: montmorillonite, kaolinite, illite and hydromuscovite. Chemical composition of the initial sample is presented in *Table 1*.

Table 1. *Chemical composition of the initial bentonite sample*

	Bentonite						
Chemical composition	SiO_2	Al_2O_3	K_2O	Fe_2O_3	TiO_2	MgO	CaO
Content, %	67.81	24.73	3.25	1.89	1.06	0.93	0.33

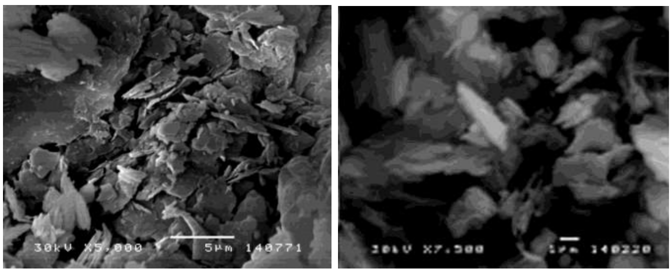

Figure 1. *SEM of unmodified clay*

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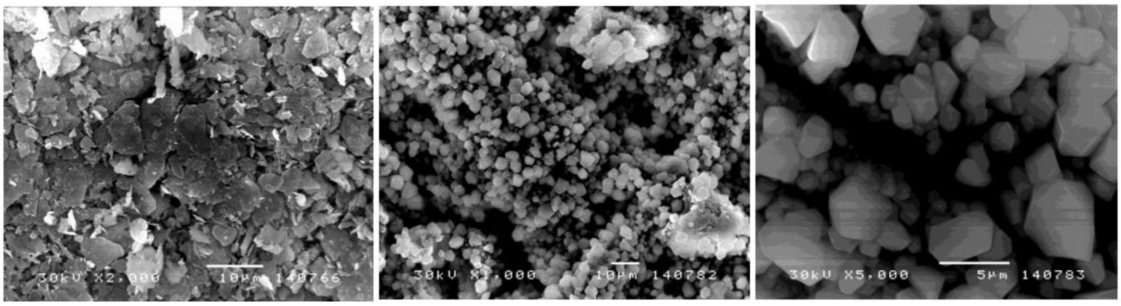

Figure 2. *SEM of clay modified with sodium carboxymethyl cellulose*

SEM analysis of unmodified clay (5000× magnification) shows characteristic morphology of clay minerals, i.e. platelet particles of different sizes (*Fig. 1*). SEM analysis of GI sample indicates lamellar particles with dominant fraction of 5-10 µm in diameter. Microphotographs of GII sample show polyhedral crystals, which are more evident on SEM analysis of GIII sample. Having on mind that there are no published publications of clay modification with NaCMC, we assumed these crystals originated from NaCl were formed in the reactions of HCl and sodium from NaCMC (*Fig. 2*).

SEM-EDS analysis shows that predominant elements in unmodified clay are: Fe, Al, Si, and O. In sample of GI, GII and GIII aluminum is the most dominant, due to leaching of Fe during the synthesis of composite material.

FTIR spectral analysis

FTIR spectra of modified clays (GI, GII, GIII) (*Fig. 3*) show OH band at 3430.65 cm^{-1}, 3433.42 cm^{-1}, 3433.87 cm^{-1} succesively characteristic for clay minerals (montmorillonite, illite, hydromuscovite and kaolinite).

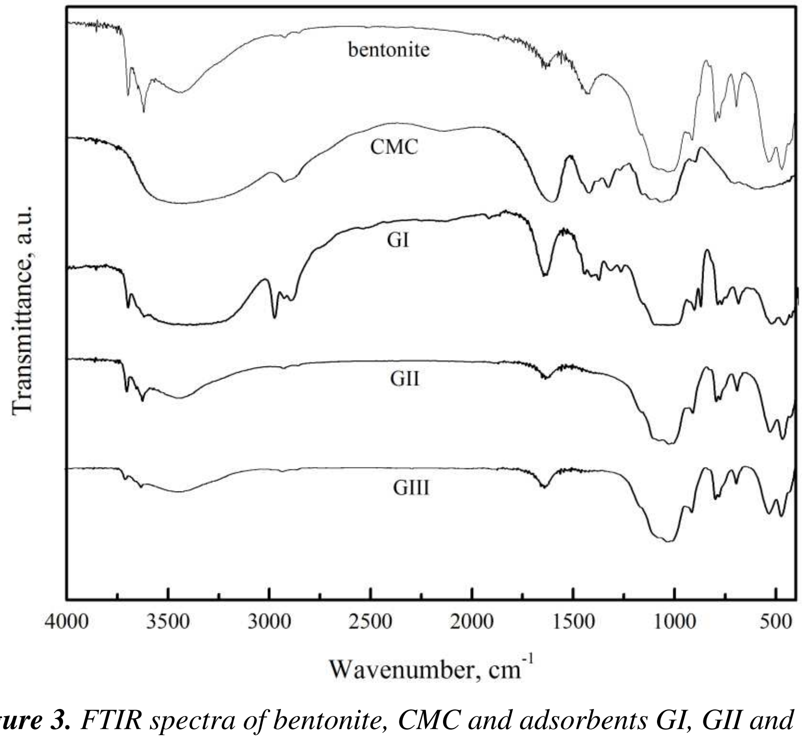

Figure 3. *FTIR spectra of bentonite, CMC and adsorbents GI, GII and GIII*

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Also, in spectra (G0, GI, GII, GIII) it is clearly visible the intensive band coming from deformational vibration of Si-O bond, with maximums at 1033.27 cm^{-1}, 1028.52 cm^{-1}, 1037.13 cm^{-1} and 1019.46 cm^{-1} (characteristic for alumosilicates present in the clay). At the spectrum of modified clay GI, peaks at 2926.41 and 2854.77 cm^{-1} come from C-H vibrations and they are results of the presence of carboxymethyl cellulose. In spectra of GII and GIII, these bands are less pronounced, which means that sample GI contains mostly unmodified carboxymethyl cellulose, although in the process of the preparation the least quantity of this organic compound as a modifier of the surface and adsorption characteristics of clay was used.

Adsorption experiments

Influence of pH on the adsorption

Lead adsorption (50 mL of the Pb^{2+} solution, concentration 100 mg L^{-1}) on modified clay (1.00 g) was investigated at pH values 1, 3, 5, 7 and 9. At *Figure 4,* it was given the dependence onadsorption capacity of adsorbent for Pb^{2+} ions (mg g^{-1}) as a function of pH of the environment. It is obvious that the adsorption increases with the increase of pH value. In strong acidic environment, the number of H_3O^+ ions is higher that the number of Pb^{2+} ions in the solution, so at low pH values the active places for the adsorption are occupied with H_3O^+ ions which unable the adsorption of Pb^{2+} ions (Bhattacharyya and Gupta, 2007). Qiu et al. (2008) noticed the existence of lead in different forms at different pH, such as Pb^{2+}, $Pb(OH)^+$, $Pb(OH)_2$, $Pb(OH)^{3-}$, $Pb(OH)_4^{2-}$, etc. At pH=6.7, the precipitation of lead in the form of hardly soluble $Pb(OH)_2$ occurred, so the decrease of the concentration in this pH range can not be attributed to adsorption (Qiu et al., 2008). In any case, it is evident the increase of the percentage of removed lead by adsorption at pH values up to 7. Similar results were obtained by Bhattacharya and Gupta (2007).

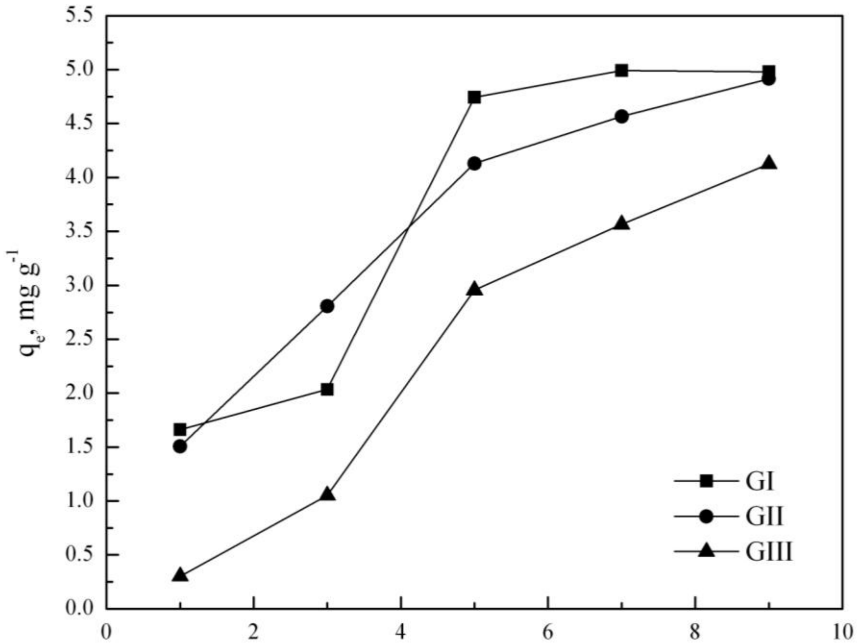

Figure 4. *The influence of pH on the adsorption of Pb^{2+} ions*

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Modified clay GI is the most sensitive to pH change which increases the possibility of its application.

The influence of temperature on the adsorption

The influence of the temperature on the adsorption of lead (50 mL of Pb^{2+} solution, c=100 mg L^{-1}) on modified clay (1.00 g) was investigated at five different temperatures (20 ^{0}C, 30 ^{0}C, 40 ^{0}C, 50 ^{0}C and 60 ^{0}C). From *Figure 5*, it is obvious that the increase of temperature causes the increase of Pb^{2+} adsorption from the solution, indicating that the adsorption of lead on the investigated adsorbents is an endothermic process. This increase is the most pronounced in case of sample GI.

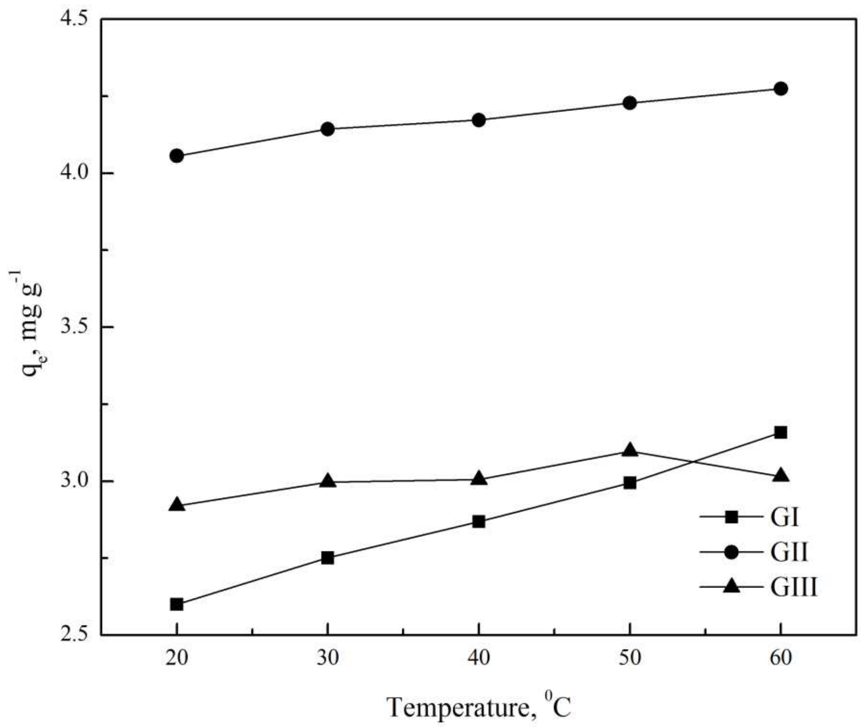

Figure 5. *The influence of the temperature on the adsorption of Pb^{2+} ions*

The influence of the mass of the adsorbent

The influence of the mass of the adsorbent on the adsorption of Pb^{2+} ions (50 mL of Pb^{2+} solution, c=100 mg L^{-1}) was investigated with the following weights of modified clay: 0.25, 0.50, 1.00, 1.50 and 2.00 g. Results are shown in *Figure 6*. It is obvious that increasing of mass of the adsorbent causes the increase of the percentage of adsorbed lead since the adsorption is carried out on the available surface, so for the solution of fixed concentration, the increase of the quantity of adsorbent gives the higher area i.e. more places for the adsorption (Chaari et al., 2008).

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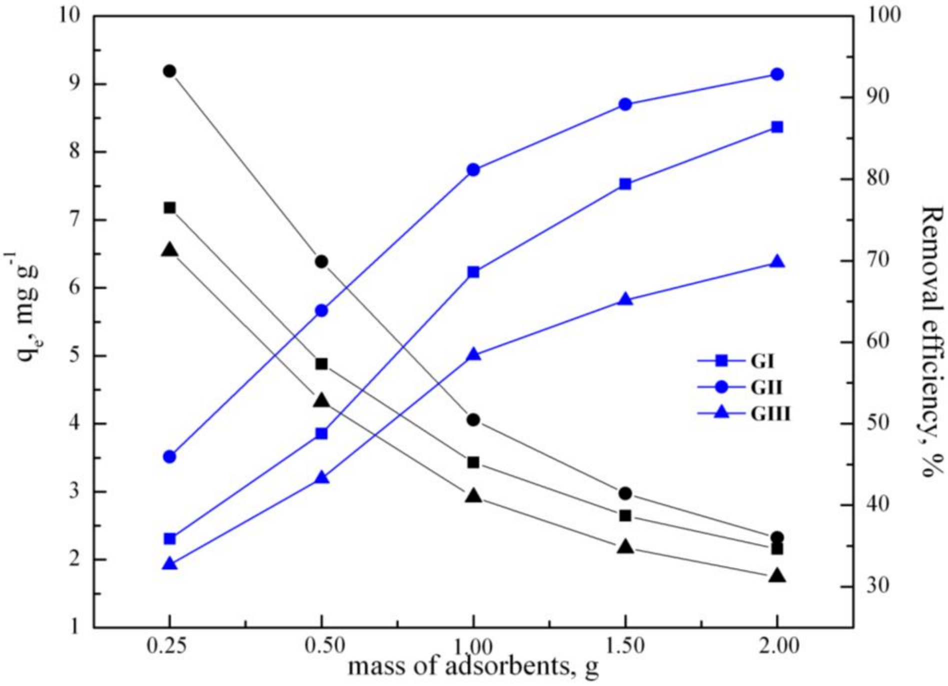

Figure 6. *The influence of the mass of the clay on the adsorption of Pb^{2+} ions*

The highest effect of the removal of Pb^{2+} ions was shown by the modified clay GII.

50 mL of the solution of different initial concentrations Pb^{2+} was added to 1 g of the modified clay. pH of solution was 5.0, and experiments were performed at 20 ºC (*Fig. 7*).

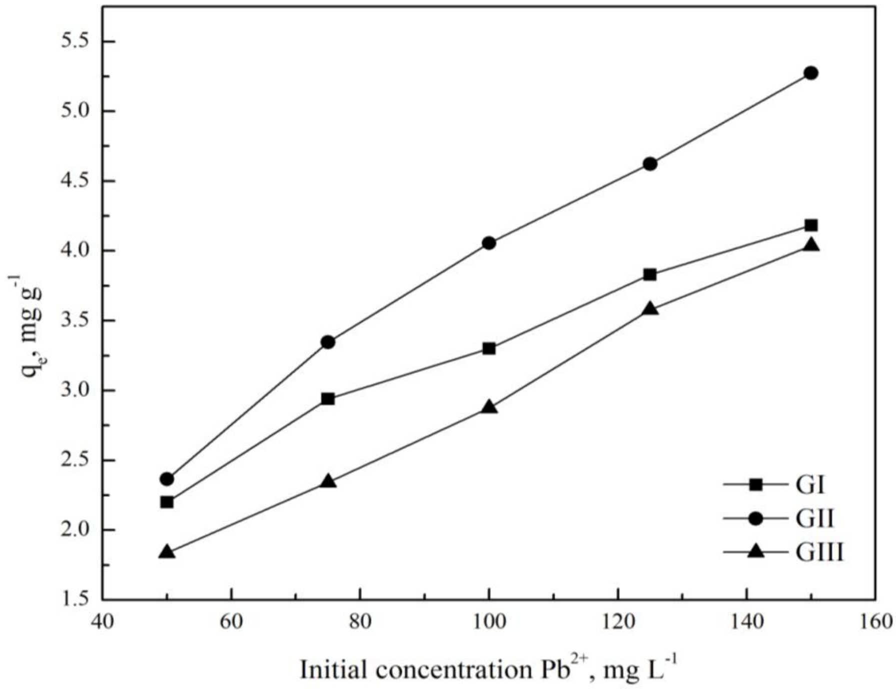

Figure 7. *The influence of the initial concentration of adsorbate on the adsorption of Pb^{2+} ions*

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Adsorption capacity of clay (adsorbate) GII is the highest and increases with increasing of initial concentration of Pb^{2+}, while the smallest is for clay (adsorbate) GIII. The highest percentage of added NaCMC (5%) is the reason of minimum adsorption capacity. The results show that under the same experimental conditions, adsorbate with 3% of NaCMC is the most efficient (with highest adsorption capacity). This can be explained by the formation of the most suitable structure with the addition of NaCMC in concentration of 3%. The addition of polymer onto clay and improving the adsorption properties is also reported other authors (Stojiljkovic et al., 2014).

Adsorption isotherms

Based on the obtained results, Langmuir and Freundlich adsorption isotherms were applied for the investigation of modified clay.The Langmuir model assumes that the uptake of metal ions occurs on a homogeneous surface by monolayer adsorption without any interaction between adsorbed ions (Langmuir, 1918). The Langmuir parameters can be determined from a linearized form, by plotting Ce/qe vs. Ce (*Fig. 8, Eq. 2*):

$$\frac{c_e}{q_e} = \frac{1}{K_{Lq\max}} + \frac{1}{q_{\max}} c_e \quad \text{(Eq. 2)}$$

where C_e is the equilibrium concentration of adsorbate (mg dm^{-3}), q_{max} is the Langmuir equilibrium constant related to maximum monolayer coverage capacity (mg g^{-1}), and K_L is the Langmuir constant which is related to the enthalpy of adsorption (dm^{-3} mg^{-1}).

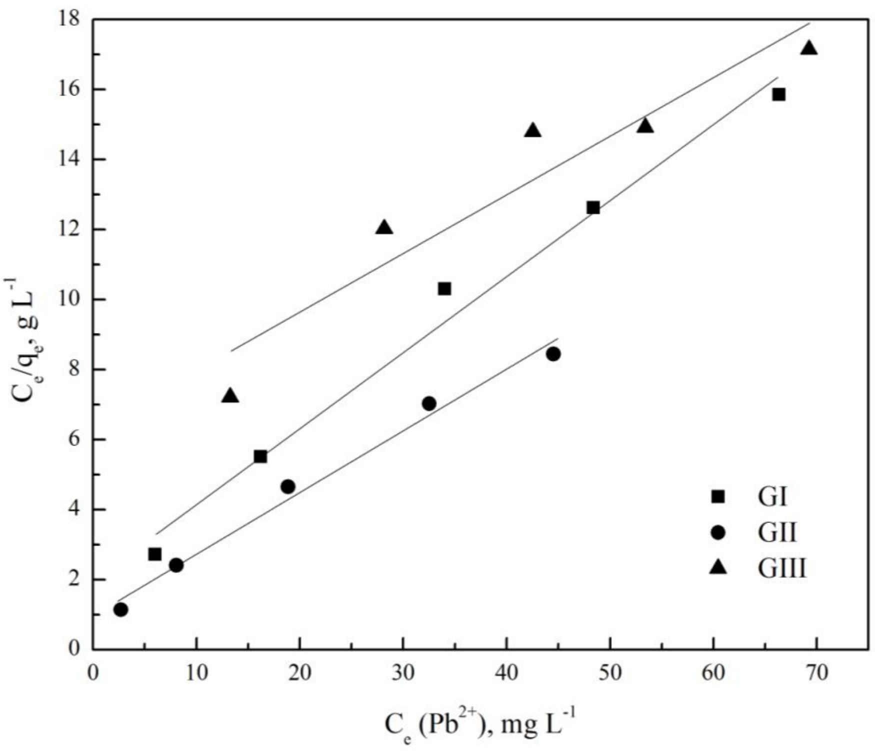

Figure 8. *Langmuir adsorption isotherms for the adsorbents GI, GII and GIII*

Based on logarithmic form of Freundlich equation (*Eq. 3*):

$$\log q_e = \log K_f + \frac{1}{n}\log c_e \qquad \text{(Eq. 3)}$$

where q_e is the adsorption capacity (mg/g), K_f Freundlich constant, 1/n Freundlich exponent and c_e concentration of Pb^{2+} ions in the equilibrium (mg dm^{-3}), the plot of the log q_e against log c_e is presented (*Fig. 9*).

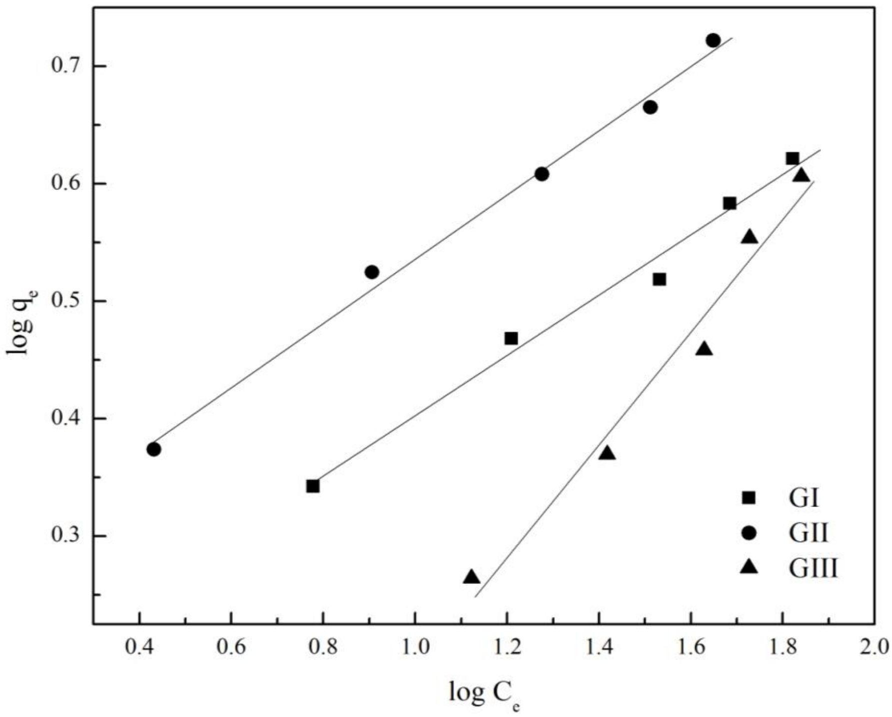

Figure 9. *Freundlich adsorption isotherms for the adsorbents GI, GII and GIII*

In *Table 2* parameters of Langmuir and Freundlich adsorption isotherms were given for the investigated adsorbents.

Table 2. *Parameters of Langmuir and Freundlich adsorption isotherms for the investigated adsorbents*

Adsorbents	Freundlich			Langmuir		
	K_f	n	R^2	K_L	Q_{max}	R^2
GI	1.3890	3.8585	0.98159	0.1099	4.6024	0.98232
GII	1.8256	3.6387	0.99181	0.1789	5.6860	0.98325
GIII	0.5108	2.0849	0.96289	0.0266	5.9687	0.87946

Results obtained by this study are similar with results for clay modification with polymers (Stojiljkovic et al., 2014). This is not surprising because both NaCMC and polymers, which are used to modify clay, are long chain molecules that are expected to increase adsorption process.

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Conclusions

Suitability of the clay modified with NaCMC for the removal of toxic heavy metal (lead) from the aqueous solution was tested in adsorption studies. The effect of various factors, such as the effect of pH of the solution, temperature, quantity of the adsorbent, concentration of the adsorbate was determined in a series of the experiments. It was found that the adsorption, i.e. the percentage of removed lead increases with the increase of these parameters. Obtained Freundlich adsorption isotherms show extremely high correlation coefficient R, i.e. processes of equilibria on the investigated clays can be described using this equation. The series of conducted experiments shows that the clay modified with carboxymethyl cellulose is suitable for the removal of lead from waste waters. Removing metal ions from aqueous solutions by adsorption onto bentonite and its modifications is efficient and cheap technique. Further researches should be aimed to test adsorption properties of the clay modified with other long chain molecules, and to test its efficiency in removing heavy metal ions from water solutions in different conditions.

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RESPONSE OF TISSUE CULTURED BANANA (*Musa acuminate* L.) cv. GRAND NAINE TO DIFFERENT LEVELS OF NUTRIENTS UNDER DRIP FERTIGATION AND BLACK PLASTIC MULCH

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Abstract.A field experiment was carried out in two crop seasons in the lateritic sandy loam soils of Kharagpur, West Bengal, India, to investigate the response of banana (*Musa acuminata* L.) cv. Grand Naine at different levels of nitrogen, phosphorous and potassium nutrients applications through drip fertigation and plastic mulch. A randomized complete block design was used with four fertigation levels in conjunction with mulch and without mulch. Fertigation levels caused a significant increase in fruit yield and determined the response to N, P and K fertilizers. The results of recommended dose of fertilizers application through drip either alone or in conjunction with black plastic mulch conditions were compared with other fertigation treatments in terms of growth and crop of yield. Both the main and ratoon crops performed best for 80 per cent of the recommended fertigation dose (160 N: 48 P: 240 kg plant^{-1} year^{-1}) covered with plastic mulch in respect of (a) growth parameters; maximum plant height, stem girth, functional leaves, yield parameters and shortened total crop duration for 34 days and for (b) quality parameters; higher levels of TSS, reducing sugar and non-reducing sugar, pulp:peel ratio and lower content of acidity. Hence, fertigation with 80 per cent of the recommended dose coupled with plastic mulch was found to be optimum and economical for banana cultivation.
Keywords: *crop coefficient; nitrogen; potassium; nutrient conversion efficiency; B-C ratio*

Introduction

Banana is a fast growing plant that requires high and continuous nutrient and water supplies to sustain a yearlong cycle and ensure economically high yield. These nutrients may be partly supplied by the soil, but fertilizer application is generally needed to satisfy the needs of theplant and to obtain profitable production. In almost all thefertigation studies, researchers suggested high nutrition (nitrogen, phosphorous, potassium) requirement of banana crop (Lahav, 1995). According to Lahav (1995), a banana crop yielding 50 t ha^{-1} requires about 390 of N, 50 of P and 1440 of K (kg ha^{-1}).

The increasing fertilizer costs and the demand have emphasized the need for full exploitation of sources of nutrientson a global scale. So in today's perspective, it is essential, especially in crops like banana which are the heavy feeder of nutrients it is desired to use efficien tmethod of fertilizer application and splitting the dose to get maximum fertilizer use efficiency (FUE) and net profit. Under sub-tropical conditions, soil nutrients will be leached rapidly due to various factors and therefore it should be applied in small doses at shorter intervals. In recent years, liquid fertilizers are used as astrong alternative to solid fertilizers. The major advantage of liquid fertilizers is that, they are completely soluble in water and can be applied through drip system with an ease, without any harm and in number of splits. The second strong point goes in favour of liquid fertilizeris that they contain major essential nutrients in readily available form. In the fertigation process predissolved soluble fertilizers are injected into the feeder line

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of drip irrigation system. Fertigation increases the nutrient use efficiency by 30-40%, prevents soil degradation, reduced the cost of fertilizer application and minimizes the pollution of groundwater, also preventing losses through runoff and leaching. It ultimately increases the FUE by increasing the nutrient uptake and minimizing the losses. It also improves the productivity andquality of the produce (Srinivas, 1999; Mahalakshmi et al., 2001; Pandey et al., 2001).

Mulches also contribute to the crop productivity by way of influencing moisture content, soil productivity, weed control etc. (Sweeney et al. 1987). Use of different types of soil covers ormulches like straw, leaves, husk, crop residues and black plastics have been found to conserve moisture, nutrients, control weeds, moderate soil temperature and increase in yield ofdifferent vegetables. It is observed from studies that beneficial response of plants to mulch includes earlier production (Call and Courter, 1989), greater total yield and reduced insect and disease problems (Greenough et al., 1990). The yield response of plastic mulch in vegetable and fruit crops is reported to be greater than organic mulches (Tiwari et. al., 1998a,b; Tiwari et. al., 2014). The combined effect of fertigation and mulch on banana crop yield has not been studied for sub humid climatic and lateritic soil condition.

The objective of the present study is to investigate the response of different levels of fertigation through drip and black plastic mulch on yield and nutrient use efficiency of banana crop. It is alsoproposed to investigate the effects of cropping on chemical properties of soil.

Materials and Methods

Experiment was conducted at the Experimental Farm area of Precision Farming Development Centre Project, Agricultural and Food Engineering Department, Indian Institute of Technology, Kharagpur, India. The experimental site is located at flat land with latitude of 22°18.5' N, longitude of 87°19' E and an altitude of 48 m above mean sea level. The local climate is sub-humid subtropical with an average annual rainfall of 1390 mm, of which about 80% is received during June to October. The mean monthly minimum temperature is 6 ^{0}C in January, whereas the mean monthly maximum temperature is 43.5 ^{0}C in May. The mean monthly relative humidity varies from 35% in February to 96% during July-August. The soil of the experimental area is lateritic with sandy loam texture.

The experiments were laid out with tissue cultured banana cv. Grand Naine as a test crop in randomized block design (RBD) with eight treatments and three replications (*Figure 1a*). Each treatment had a net area of 2 m × 15 m with 15 plants in each treatment. Banana was planted on raised bed and four treatments (12 rows) of crop bed. The bed was covered with black plastic mulch of 50 micron thickness of 1 m wide and 10 m long for each row (*Figure 1b*). The details of the treatments are furnished below.

T_1 (1.2 RDF + M):	120% of fertilizer requirement application through drip and plastic mulch
T_2 (1.2 RDF):	120% of fertilizer requirement application through drip and without mulch
T_3 (RDF + M):	100% of fertilizer requirement application through drip and plastic mulch
T_4 (RDF):	100% of fertilizer requirement application through drip and without mulch
T_5 (0.8 RDF + M):	80% of fertilizer requirement application through drip and plastic mulch
T_6 (0.8 RDF):	80% of fertilizer requirement application through drip and without mulch
T_7 (0.6 RDF + M):	60 % of fertilizer requirement application through drip and plastic mulch
T_8 (0.6 RDF):	60 % of fertilizer requirement application through drip and without mulch

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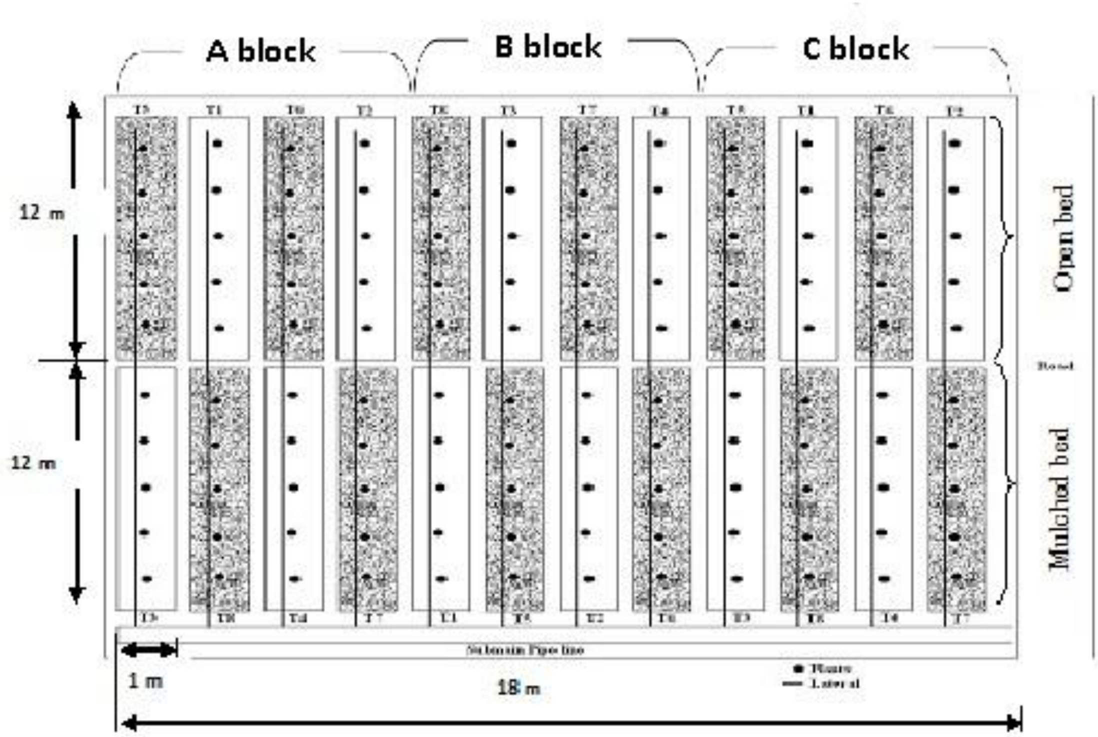

Figure 1a. *Layout of banana experimental field*

Figure 1b. *Experimental layout of banana crop with micro irrigation and black plastic mulch at PFDC experimental field, IIT Kharagpur, India*

Estimation of crop water requirement and irrigation water supply

Reference crop evapotranspiration (ET_O) was estimated using the FAO-56 modified Penmanmethod using weather data collected from Weather Station, 0.5 km away from the field site. The values of crop coefficient (Kc) suggested by Allen et al. (1998) were considered and appropriately adjusted. Soil surface evaporation component in crop evapotranspiration is considered negligible as it was covered with the black plastic mulch. The actual evapotranspiration was estimated by multiplying reference evapotranspiration with crop coefficient for different months based on crop growth stages. The daily irrigation water requirement for the banana crop was estimated using the following relationship:

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$$IR = ET_0 \times K_c - R_e \quad \text{(Eq. 1)}$$

where:
IR Net depth of irrigation (mm day^{-1})
ET_o Reference evapotranspiration (mm day-^{1})
K_c Crop coefficient
R_e Effective rainfall (mm day^{-1})

The net volume of water required by the plant can be calculated by the relationship

$$V = IR \times A \quad \text{(Eq. 2)}$$

where:
V Net volume of water required by a plant (L day^{-1} $plant^{-1}$)
A Area under each plant (i.e. spacing between rows, m x spacingbetween plants, m)

The reference evapotranspiration was estimated using FAO 56 method. The crop coefficient K_c was estimated considering the local soil condition. The effective rainfall is the part of the rainfall that forms the part of the consumptiveuse. The irrigation water was supplied after subtracting the effective rainfall from the total irrigation requirement (*Eq. 1*).The water requirement was estimated for the main and ratoon crop of banana using *Eq. 2*. The irrigation water was applied for the entire crop season as per the crop growth stages using drip irrigation (*Figure 2a*).

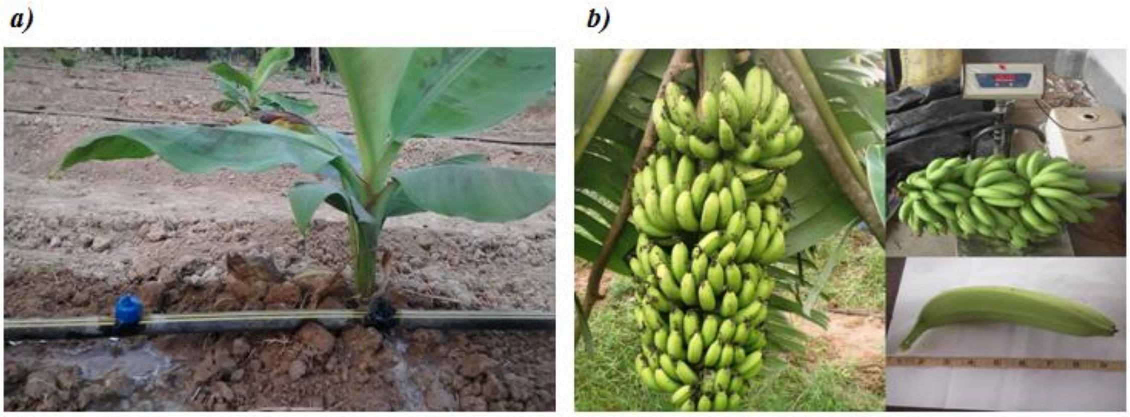

Figure 2a*. Application of fertilizer for banana crop using micro irrigation*
Figure 2b*. Weighing banana bunch and measuring length of banana fruit*

Fertigation schedule application

The dose of fertilizer for Grand Naine was considered as 200:60:300 N:P_2O_5:K_2O $kgha^{-1}$as recommended by Mahalakshmi et al. (2001) and Nalina et al. (2000). This recommendation is for manual application. Using this recommended values weekly schedule was estimated considering the nutrients requirement at different growth stages of plant as given in *Table 1*. Water soluble fertilizers were applied twice in a week through drip irrigation system.

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Monitoring the growth parameters

Growth parameters monitoring under each treatment included seasonal increase in plant height (5 cm from the base of thepsuedostem to the point of interaction of the petioles ofthe two youngest leaves), plant girth (5 cmabove the ground levels), the number of green leaves, and leaf area (Murray, 1960). Mature bunches were harvested when they reached full three quarter shape. Yield and yield components were recorded under each treatment (*Figure 2b*). The second hand of freshly harvested bunch was used to measure the fruit quality analysis (Pulp:peel ratio, TSS, Total sugar, Acidity) as suggested by Dadzie & Orchard (1997).

Table 1. *Fertilizer recommendation for banana crop*

Crop stage (No. of weeks after transplantation)	Quantity (N:P:K), g/plant	Schedule ((N:P:K), g/plant/fertigation*
Basal Dose	1 kg Neem Cake,+ 20 FYM	While Transplanting
Initial stage (1 to 8)	58:30:30	3.6: 1.9: 1.9
Developmental stage (9 to 16)	90:30:90	5.6: 1.9: 5.6
Fruiting stage (17 to 24)	32:00:100	2: 0: 6.3
Harvesting stage (25 to 40)	20:00:80	0.7: 0 : 2.7

*fertigation is scheduled twice in a week

Monitoring the plant chemical parameters

Third leaf laminas were sampled (after the first hand of male flowers could be seen onthe inflorescence) as recommended by Martin-Prével (1992); samples from bunch stalkand fruits were also taken and their nutrient concentrations were determined. The plant parts were dried at 80°C for 48 hours and the concentrations of N (Kjeldahl method), P (Spectro colorimeter) and K (flame photometer) were measured. The amountof nutrient in each organ was calculated from dry weight and nutrient concentration.

Statistical analysis was performed using SPSS software package to test the significance of different treatments individually as well as in combinations experimental ANOVA was performed by the method described by Gomez and Gomez (1984). In order to the comparisons between the treatment means and variance were tested at 5% significance level. Duncan multiple range test was conducted to know the significance level between the treatments and groups of treatments.

Results and Discussion

Estimated water requirement for banana crop

Reference crop evapotranspiration (ET_o) was estimated using the FAO-56 modified Penman method using weather data. The actual evapotranspiration was estimated by multiplying reference evapotranspiration with crop coefficient based on crop growth stage. In present study the value for daily crop coefficient (Kc) was estimated using dual crop coefficient method. Summation of both Kcb and Ke curve makes crop coefficient (Kc) curve on daily basis for banana crop. The soil evaporation coefficient (Ke) was considered zero when soil is covered with plastic mulch. Therefore the value of Kcb is

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considered as Kc for calculation of crop evapotranspiration under mulch cover. The ground cover reduction factor for banana crop under drip irrigation started from 0.1 and maximum ground cover estimated is about 0.96 at the developmental stage. The daily irrigation water requirement for the banana crop was estimated by subtracting the effective rainfall from the calculated evapotranspiration. The estimated quantity of water applied to banana plants for main and ratoon crop was 1236, 612 mm for without mulch and 1115 mm, 520 mm for plastic mulch treatment respectively. *Figure 3* shows the month wise distribution of banana crop water requirement for the tissue cultured banana plantlets transplanted in October month. The total crop duration was 11-12 months for main crop and 10-11 months for ratoon crop. The crop water requirement of banana varies from 1.59 to 19.6 L day^{-1} $plant^{-1}$ from early stage to peak demand period without mulch and 0.66 to 17.71 L day^{-1} $plant^{-1}$ for plastic mulch covered condition (*Fig. 3*). The total quantities of water applied to banana plants were 3.43 m^3through drip irrigation system without plastic mulch coverand to 2.59 m^3 for drip with plastic mulch covered condition.

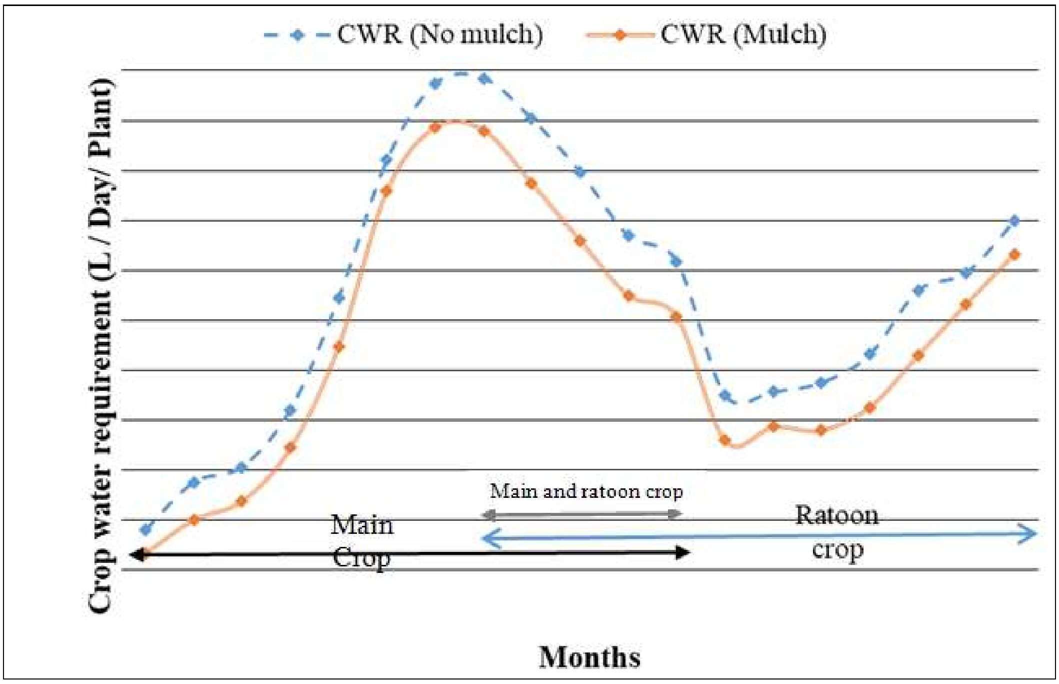

Figure 3. *Estimated crop water requirement of banana in open and plastic mulch conditions*

Effect of fertigation levels on biometric properties of banana crop

Treatment wise biometric observations of the crop were recorded and after analysis of data these are given in *Table 2*. Pooled data over two crop season indicates that vegetative growth parameters were significantly influenced by different level of fertigation treatments. *Table 2.* shows the values of biometric attributes (plant height, stem girth, no. of functional leaves and leaf area) under different treatments. The results showed that thetreatments differences were very highly significant forplant height, no. of functional leaves & significant for leaf area of the main plant and ratoon crop of banana cv. Grand Nain. The best growth parameters were obtained at 80% of RDF covered with plastic mulch (T_5) followed by 100% RDF covered with plastic mulch (T_3).

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The result indicated that there was a significant difference among fertigation treatments in pseudo stem height and stem circumference or girth. The stem height in all the treatments showed steady increase throughout the growt hcycle of the first and ratoon banana plants. The treatment T_5 recorded the maximum plant height (207.1&183.8) in comparison to other treatments, which was statistically at par with treatment T_3 80% of recommended dose of NPK through drip irrigation and soil covered with plastic mulch, which registered a plant height of (201.6 & 178.3) in first and ratoon crop respectively. While the treatment T_8 had the lowest stem height (171.0&153.4) in first and ratoon crop respectively. Growth response of banana to NPK fertilization through drip irrigation (fertigation) indicated that the T_5 (80% RDF + mulch) treatment responded best performance in terms of growth in main crop under study. These results are almost similar with the data reported by Belalcazar and Espinosa (2000). Increased uptake of nutrients particularly nitrogen under fertigation system could be attributed to enlarge of stem height. The absorbed nitrogen ultimately might have been utilized by the plants in the formation of complex substances like protein and amino acids which in turn help to build up new tissues (Childers, 1966). These results are in accordance with the findings of Martinsson et al. (2006) who obtained maximum plant height in starw berry for optimum dose of fertilizer application through drip.

Table 2. *Response of fertigation and plastic mulch on biometric properties of plants*

Treatment	Pseudostem height at shooting (cm)		Pseudostem girth at shooting (cm)		Functional leaves up shooting (No)		Leaf area (m^2)	
	First Crop	Ratoon Crop	First Crop	Ratoon Crop	First Crop	Ratoon Crop	First Crop	Ratoon Crop
T_1	187.0 cd	157.0 cd	55.9	44.5 c	11.3 d	10.7 e	0.9 cd	0.91 cd
T_2	176.0 de	156.0 cd	54.9	43.5 c	10.3 de	10.3 e	0.9 d	0.82 de
T_3	201.6 ab	178.3 ab	57.0	48.7 b	13.7 b	12.7 b	1.2 ab	1.08 ab
T_4	189.1 bcd	164.6 cd	56.5	46.5 bc	12.3 c	11.7 c	1.0 bc	0.94 c
T_5	207.1 a	183.8 a	60.6	52.2 a	15.0 a	13.7 a	1.3 a	1.1 a
T_6	193.3 bc	167.7 bc	58.5	46.8 bc	12.7 bc	12.0 bc	1.1 b	1.05 b
T_7	181.1 cde	156.1 cd	53.6	43.3 c	10.7 e	10.3 e	0.9 cd	0.89 cde
T_8	171.0 e	154.4 d	53.8	43.2 c	10.0 e	10.0 e	0.8 d	0.81 e
Sem ±	4.94	4.02	NS	1.17	0.31	0.29	0.04	0.03

* Any two means having a common letter are not significantly different at the 5% level of significance

Pooled mean values of two crop season showed that fertigation with 80 per cent RDF through drip irrigation and plastic mulch (T_5) recorded highest stem girth (60.6 and 52.2 cm) in main and ratoon crop respectively. It was followed by the treatment T_3 (57 & 48.7 cm) and the lowest value was recorded in T_8 (53.8 & 43.2 cm) in main and ratoon crop respectively. Effect of fertigation level on stem girth in main crop was not statistically significant. However, effect of treatment was significant in ratoon crop. This may be due to plastic mulch which influences the emergence and growth of suckers. Hegde and Srinivas (1991) also had similar observations as in the present study which indicated that increasein amount of nitrogenand potassium fertilization had no significant effect on stem girth effect. This is perhaps due to sufficient amounts of soil NPK 80% RDF maintained adequate vegetative growth in this study.

A banana crop should produce sufficient number of leaves to harness thelight energy and synthesize adequate photosynthates for biomass production. The role of leaf parameters such as number of leaves produced and number of functional leaves retained at shooting is crucial in determining the yield potential.The fertilizer treatment had little effect in producing number of leaves. The leaf number increased slightly in response to the fertigation treatments. At the time of flowering, all the treatments produced the same number of leaves (ranged from 10-14 leaf/plant). This exceeded the lowest number of leaves (eight leaves) required at flowering to obtain high yield (Martinez, 1984). The application of 80% NPK per plant and plastic mulch (T_5) significantly enhanced the number of leaves at all stages of growth both in plant and ratoon crops which indicated that the application of NPK at optimum rate promoted faster rate of leaf production.

Apart from the number of leaves, leaf area at any growth stage is very critical for banana crop as it has a close bearing on photosynthetic efficiency reflecting on biomass production. An increase in leaf area results in better utilization of solar energy. For individual leaf, treatment T_5 had the largest leaf area followed by T_3 which is at par with treatment T_5.

The increased height, girth, number of leaves and leaf area in mulched treatments corresponding with same fertigation level was recorded. This may be attributed to sufficient soil moisture near root zone and minimized leaching of nutrients due to mulching. Similar findings have also been obtained by Ansary and Roy (2005) in water melon, Al-Majali and Kasrawi (1995) in muskmelon, Hallidri (2001) in cucumber.

The maximum number of suckers were recorded in the treatment T_3 (100 % RDF with plastic mulch) as 10.7 in the main crop and 4.3 in the ratoon crop. Treatment T_5 (80% RDF covered with plastic mulch) took 336& 305.7 days to harvest main and ratoon crop respectively which was lesser than other treatments (*Table 3.*). Crop duration or is an important factor to be considered in banana cultivation especially under fertigation through drip. The crop duration can be divided into two phases viz., days taken from planting to shooting in main crop or sucker setting to shooting of ratoon crop and days taken from shooting to harvest. In the present experiment, it was revealed that optimum dose of fertigation through drip with plastic mulch caused reduction in crop duration of main and ratoon (*Table 3*). From these experimental results, it appears that the main crop grown under treatment T_5 shooting flower nine days earlierthan treatment T_4 (100 % RDF with no mulch), while in the ratoon crop eight days early flower emerges from the treatment T_5 compare to the treatment T_4. The early shooting under the treatment (T_5) could be attributed to the rapid production of leaves, which would have elaborated more photosynthates and increased flowering stimulus.

Normally, higher fertigation and irrigation levels cause excess vegetative growth that may delay in flowering period. The delay in shooting and harvest in the treatments T_1 and T_2 may be due to increased nitrogen status which might have been causedby the supra optimal level of nutrients leading to poor development of root system which also reflected in lesser number of roots. When the root number is less, it may notbe adequate to meet the nutrients required by the aerial parts. This is in agreement with Nalina et al. (2000) whofound that the crop supplied with optimum dose of fertigation needs less number of days to shooting (226 days) and total crop duration (338 days). Mahalakshmi (2001) also noted early shooting, early bunch development including total crop duration under low levels of nutrients and water. This can be explained due to the better source sink relationship in treatment T_5 which recorded more leaf area with more photosynthetic activity, translocating the carbohydrate efficiently to the development of bunch.

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Table 3.*Effect of fertigation & plastic mulch treatments on number of suckers, days taken from flowering to harvest*

Treatment	Suckers (No)		Days to flowering (days)		Days to harvesting (days)	
	First Crop	Ratoon Crop	First Crop	Ratoon Crop	First Crop	Ratoon Crop
T_1	8.3 ab	2.7	269.7 b	275.7 bc	358.7 c	335.3 cd
T_2	5.7 d	3.3	281.3 c	281.3 c	374.0 de	350.7 e
T_3	9.3 ab	4.0	258.0 a	263.3 ab	341.0 a	318.0 b
T_4	6.7 bc	3.7	266.0 ab	269.7 bc	356.7 c	331.7 cd
T_5	9.7 a	4.3	258.3 a	251.7 a	342.7 ab	305.7 a
T_6	6.7 bc	4.0	261.0 ab	270.0 bc	354.0 bc	330.3 c
T_7	8.0 c	3.3	280.0 c	276.7 c	371.0 d	340.0 d
T_8	4.3 c	2.7	292.7 d	278.7 c	386.0 e	356.3 e
Sem ±	0.44	0.26 NS	3.74	6.05	4.25	2.71

* Any two means having a common letter are not significantly different at the 5% level of significance

Yield and yield component are the most important economic traits, which are very highly influenced by fertigation treatments. Yield in banana is a function of bunch weight and number of plants perhectare. In the present study, the most important economic trait, namely bunchweight was significantly influenced by the various fertilizer treatments (*Table 4*). The maximum yield and bunch weight was obtained T_5. The number of hands andfingers per bunch with treatmentsT_5, T_3, T_6 and T_4 were significantly greater than others fertigation treatments of the first and ratoon crop (*Table 4*). The bunch weight decreased when level of N, P and K fertilizer doses decreased T_7 and T_8 also increased doses at T_1, T_2, T_3 and T_4. The results also showed thatthe higher dose of fertilizer application (as in T_1& T_2) mayresult in small bunch weight. This was also observed by Bolanos et al. (2003) where excessive potassium applications reduced banana yield.

The increase in yield (bunch weight) was attributed toincrease in the total fruit weight. Highest yield was recorded in treatment T_5 and yield of treatment T_3 is at par with the yield T_5 treatment (64.49 & 50.07 t ha^{-1}) in both main and ratoon crop. Remaining treatments show significant difference in yield. Similar findings were observed by Srinivas (1999), while studying the effect of N, P and K fertilizers on banana cv. Basrai. Kohli et al. (1976) reported that the nitrogen application significantlyinfluenced the fruit yield in both the crops (plantcrop, first ratoon and second ratoon).

The banana yield with drip fertigation in the presence of black plastic mulch (T_1, T_3, T_5, T_7) were significantly higher than that obtained without mulch (T_2, T_4, T_6, T_8) with respective same amount of fertilizer application. The results of the present study agreed with the finding of Igbal et al. (2009), Wang et al. (2009), and Parmar et al. (2013) who indicated that plants under polyethylene mulch produce larger fruit and have higher fruit yield per plant because of the better plant growth due to favorable hydro thermal regime of soil and complete weed free environment. Furthermore, they mentioned that the extended retention of moisture and availability of moisture also lead to a higher uptake of nutrient for proper growth and development of plants.

There was significant difference in yield with the treatment T_5 in comparison with the Treatment T_2 (120% RDF with plastic mulch). Though banana crop is known for an

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exhaustive crop amount of nutrients requiring crop at various stages of growth and development, perhaps availability of nutrientsbeyond its requirement might probably have cause its negative effect. This is also evident from the present study, where in, application of nutrients above 100 per cent did not correspondingly increased bunch weight in both plant and ratoon crops. Similar study by Bolanos et al. (2003) has shown that appropriate amount of addition of nutrients in the soil is important in obtaining higher total fruit weight.

Table 4. *Yield & yield attributing characteristics of banana under different doses of fertigation and plastic mulch treatments*

Treat-ments	Bunch weight (kg)		No of hands /bunch		Number of fingers/ hand		Finger length (cm)		Yield (t ha^{-1})	
	FC	**RC**	**FC**	**RC**	**FC**	**RC**	**FC**	**RC**	**FC**	**RC**
T1	15.32 c	16.80 abc	8.00 cde	6.33 abc	18.00 cde	12.67	19.02 bc	11.87 bc	38.30 d	40.67 bc
T2	13.95 cd	15.60 c	7.00 e	5.33 c	16.33 e	11.33	17.92 c	11.47 c	35.12 d	40.90 bc
T3	24.62 ab	19.60 ab	9.33 ab	7.33 ab	23.67 a	14.00	20.84 ab	14.37 ab	62.66 ab	47.50 ab
T4	20.70 b	17.77 abc	8.33 bcd	6.67 abc	21.00 abc	12.67	19.40 abc	13.17 abc	50.26 c	42.13 bc
T5	25.43 a	20.20 a	9.67 a	7.67 a	22.00 ab	15.00	21.55 a	15.87 a	64.49 a	50.07 a
T6	21.69 ab	18.20 abc	8.67 abc	7.00 abc	20.00 bcd	12.33	19.46 abc	12.60 abc	54.28 bc	45.80 abc
T7	11.64 cd	16.03 bc	7.33 de	5.67 bc	17.33 de	12.00	18.59 c	11.82 c	23.83 e	39.83 c
T8	10.04 d	14.70 c	7.00 e	5.67 bc	16.00 e	11.33	17.24 c	11.50 c	21.47 e	38.57 c
Sem ±	1.34	1.14	0.31	0.51	1.01	0.78	0.67	0.58	2.97	2.22
CD	4.08	3.46	0.96	NS	3.08	NS	2.06	1.74	9.03	6.73

*FC = First crop ** RC = Ratoon Crop
++ Any two means having a common letter are not significantly different at the 5% level of significance

Effect of fertigation levels on leaf and fruit nutrient concentration

Leaf nutrient concentration in banana plant provides information on the nutrient status of the plant. In banana crop cultivation practices, especially with nutrient management the optimum leaf concentration of major nutrients provides proper growth and development of banana plant. Nutrient analysis of leaf is an important diagnostic tool to verify if the crop suffers due to nutrient deficits or inadequate in amount if thecrop health status is good. Nutritional status must be in optimal concentration especially at flower initiation stage to achieve good growth and yield the leaf analysis should be done at this stage.

The nutrient content in the leaf of the present investigation both in plant and ratoon crops revealed thatthe treatmentT5 recorded higher of N,P and Kcontents over theother treatments, which indicated that the critical level of NPK was maintained (*Fig. 4*). Based on an average N content, one would getthe impression that nitrogen nutrition of banana is well above the critical level of 2.6% N in treatments T3, T4, T5 & T6 as proposed by Lahav and Turner (1983). Different researchers have proposed different

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critical levels of N (in the 3rdleaf of banana)which ranged from 1.81 to 4.0% and an average of 3.03% (Angeles et al., 1993). Detailed examination of the data, however, revealed that N content was less than 3.03 % in all the treatments except treatment T3 and T5, below this critical value photosynthesis will be affected and results in lesser yield. Theleaf P content were analyzed and compared with the critical level of 0.2% as proposed by Lahav and Turner (1983). Based on this criterion, Phosphorous content is in optimum level (>0.14%) in all the treatments. Potassium content of leaves shows optimum (4.0-5%) as given by Lahav and Turner (1983) except in Treatment T7 & T8. This signifies the need for additional application of K fertilizers for improving nutrition and high yield of banana. It is well established fact that K improves the quality of banana, in addition to its role in increasing banana production.

Removal of plant nutrients in the harvested banana fruit is one of the major considerations informulating fertilizer recommendations. *Figure 5* shows the higher nutrient content accumulated in 80% RDF covered with plastic mulch (T_5) followed by 100% RDF covered with plastic mulch (T_3).

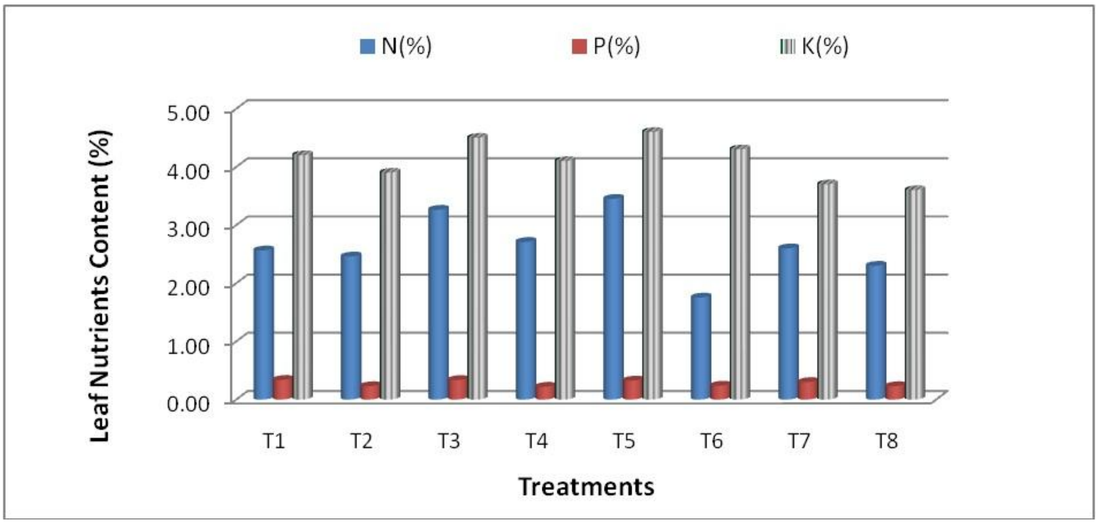

Figure 4. *NPK content of banana leaf samples in different treatments*

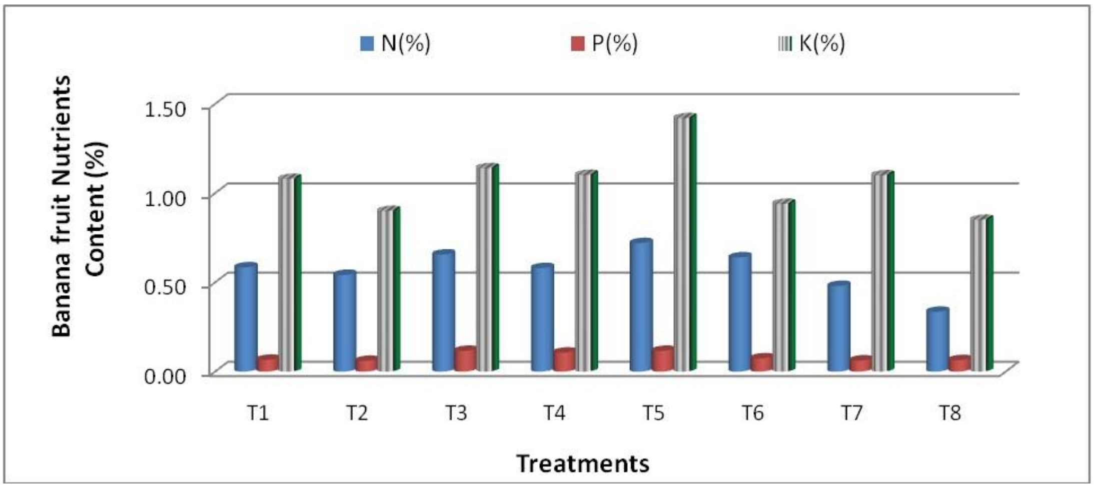

Figure 5. *NPK content of banana fruit in different treatments*

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Effect of fertigation levels on qualitative parameters of banana

In any production systems, the primary objective is to obtain maximum fruit yield per unit area without affecting the fruit quality. The fruit quality in banana is mainly judged by the sugar content and acidity in the pulp. A marked effect on fruit quality was observed with the application of adequate amount of nutrients. Higher levels of TSS, reducing and non-reducingbut a lower acidity were recorded in fruits in T_5 or T_3 (*Table 5*). Higher fruit quality especially sugar content can be explained due to the better role of nutrients particularly potassium which is involved in carbohydrate synthesis, breakdown and translocation of starch, synthesis of protein and neutralization ofphysiologically important organic acids. Further, the fruit quality parameters namely TSS and sugarswere significantly influenced by the fertigation treatments.

Table 5 shows that T_5 recorded the highest pulp: peel ratio of 2.27:1 indicating thin skin. The minimum ratio was noticed in T_8. Among the treatments evaluated, $T_5$was sweetest (24.10 ^{0}Brix) followed by T_1 (23.4 ^{0}Brix) and T_4 (23.3^{0}Brix), banana fruit analysis revealed that the treatment T_8 resulted in highest titrable acidity of 0.275%. The study reveals that N content influences TSS, sugar and acidity of banana fruit (Nalina et al., 2000). Similarly Dinesh and Pandey (2008) recorded statistically significant values of TSS, total sugars and reducing sugars with application of 75 % RDF. Hence the increased values of total sugars in banana might be due to higher uptake of nitrogen and potassium bythe plant.

Table 5. *Effect of different fertigation treatments on fruit quality characteristics of banana crop*

Treat-ments	**Pulp Peel Ratio**		**TSS (^{0}brix)**		**Reducing Sugar (%)**		**Non Reducing Sugar (%)**		**Acidity (%)**	
	FC*	RC**	FC	RC	FC	RC	FC	RC	FC	RC
T1	1.9 bc	1.96 c	23.10 bc	22.18 a	13.00 bcd	12.48 a	2.20 bc	2.11 a	0.25	0.25
T2	1.9 bc	1.83 c	22.37 c	21.47 ab	12.73 cd	12.22 ab	2.35 a	2.26 ab	0.26	0.30
T3	2.2 ab	2.47 a	23.77 ab	22.03 a	13.27 ab	12.47 a	1.84 e	1.73 a	0.25	0.27
T4	2.1 abc	2.24 ab	23.20 bc	21.85 a	13.10 bcd	12.51 a	1.94 d	1.86 a	0.25	0.25
T5	2.3 a	2.41 ab	24.10 a	22.11 a	13.53 a	12.45 a	1.76 e	1.62 a	0.24	0.24
T6	2.1 abc	2.20 b	23.43 ab	21.56 ab	13.13 abc	12.08 ab	2.12 c	1.87 ab	0.25	0.24
T7	1.9 c	1.86 c	22.77 bc	21.33 ab	12.77 bcd	11.96 b	2.30 ab	2.15 b	0.29	0.29
T8	1.8 c	1.78 c	22.37 c	20.95 b	12.57 d	11.77 b	2.40 a	2.28 b	0.27	0.27
Sem ±	0.93	0.07	0.27	0.26	0.15	0.14	0.03	0.04	0.01	0.02
CD (0.05)	0.28	0.24	0.80	0.78	0.45	0.42	0.11	0.13	NS	NS

*FC = First crop ** RC = Ratoon Crop
++ Any two means having a common letter are not significantly different at the 5% level of significance

Nutrient conversion efficiency

Drip fertigation levels had shown profound influence on nutrients use efficiency in the banana crop cultivation (*Table 6*). The maximum Nitrogen conversion efficiency of

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61.22 t kg^{-1}(ton of fruit yield to per kg of applied nitrogen), Phosphorous conversion efficiency of 537.42 t kg^{-1}, Potassium conversion efficiency of 107.48 t $kg^{-1}$and total nutrient efficiency of 57.58 t kg^{-1}were registered under water soluble fertigation through drip fertigationat 80% NPK dose with plastic mulch cover treatment (T_5) and followed by water solublefertilizers application through drip at 100% NPK dose under plastic mulch cover (T_3). The increased nutrient conversion efficiency recorded under treatment $T_5$was mainly due to better crop growth and increased yield by effectiveutilization of available nutrients that were supplied at optimum level throughout the cropperiod to meet the crop demand (Bangar and Chaudhari, 2004).

Table 6. *Nutrients conversion efficiency*

Treatment	Nitrogen use efficiency (t kg^{-1})	Phosphorous use efficiency (t kg^{-1})	Potassium use efficiency (t kg^{-1})	Nutrient use efficiency (t kg^{-1})
T_1	63.84	212.80	42.56	22.80
T_2	58.54	195.13	39.03	20.91
T_3	125.32	417.72	83.54	44.76
T_4	100.53	335.09	67.02	35.90
T_5	161.22	537.42	107.48	57.58
T_6	135.70	452.32	90.46	48.46
T_7	79.44	264.81	52.96	28.37
T_8	35.79	238.57	47.71	25.56
Sem ±	6.23	21.14	4.22	2.26
CD (0.05)	19.27	64.13	12.82	6.87

Economic analysis

The gross cost of production, net return and benefit cost ratio for different fertigation levels are presented in *Table 7.* The net returns were computed by multiplying average market rate of banana during the crop harvesting period with the yield obtained and subtracting the gross cost incurred in crop cultivation for different treatments. Maximum net profit of US $. 7199.22 per hectare and B:C ratio of 5.2 were obtained for the treatment T_5 whereas the treatment T_8 resulted in lowest net profit of US $ 3277.77 with B:C ratio of 2.7. Increase in fertigation levels causes increase in the cost of production significantly due to high cost of water soluble fertilizers as the water soluble fertilizers are generally imported in India and sold at high prices compared to straight fertilizers. Higher amount of water soluble fertilizer application in treatments T_1 and T_2 and less yield obtained compared to other treatments (T_5, T_3, T_6 and T_4) were the reason for least B:C ratio in treatments T_1 and T_2. The fertilizer levels also had significant effect on B-C ratio. The results obtained for different fertigation levels in *Table 7* are in similar trends with results reported by (Rajaraman and Pugalendhi, 2013). The mulched treatments T_1, T_3, T_5 & T_7 resulted in greater net return per ha ranging between 4% and 23% and higher B:C ratio than their corresponding treatments without mulch with drip fertigation.

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Table 7. *Cost economic of banana crop with drip irrigation and plastic mulch for different treatments*

Treatments	Gross cost of production, (US $. ha^{-1})	Yield (first & ratoon crop) (t ha^{-1})	Net return (US $. ha^{-1})	B:C ratio
T_1	1658.35	78.97	4261.44	2.6
T_2	1627.78	76.02	4070.87	2.5
T_3	1523.42	110.16	6734.45	4.4
T_4	1492.85	92.39	5432.94	3.6
T_5	1388.49	114.56	7199.22	5.2
T_6	1357.92	100.08	6144.33	4.5
T_7	1253.55	63.66	3518.56	2.8
T_8	1222.98	60.04	3277.77	2.7
CD (0.05)		8.42		0.6

Conclusions

At the current fertilizer input and farm gate banana prices, fertilizers can probably only be recommended to farmers when coupled with practices that increase soil moisture availability, such as micro irrigation and plastic mulch. Results of the present study showed that both main and ratoon crops, 80 per cent of the recommended fertigation dose (160 N: 48 P: 240 kg plant^{-1} year^{-1}) covered with plastic mulch (T_5) performed well in respect of growth parameters; maximum plant height, stem girth, functional leaves, yield parameters and shortened the total crop duration. Higher levels of TSS (24.1 ^{0}brix), reducing (13.53%) and non-reducing sugar (1.76%), pulp: peel ratio (2.34:1) but a lower acidity (0.25 %) were recorded in fruits in treatment T_5. The highest nutrient conversion efficiency (57.58 t/kg nutrient) was obtained with treatment T_5 and the lowest was (20.91 t/kg nutrient) with 120 % RDF without mulch cover (T_2). Hence, fertigation with 80 per cent of the recommended dose with plastic mulch was found to be optimum and economical.

Fertigation schedule comprises of optimum dose of nutrients and suitable frequency of nutrient applications. The present study determined the optimum dose of nutrients for banana crop in sandy loam soils and nutrient conversion efficiency under fertigation and plastic mulch. More research will be needed to know the suitable frequency of nutrients application based on climatic conditions and phenological stages of the crop. Comprehensive study required for better understanding of the effect of types of fertilizers, soil physical properties, soil chemical properties and moisture stress onnutrient uptake by banana plants, its yield and physical and chemical properties of banana fruits.

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STABLE ISOTOPES OF HYDROGEN AND OXYGEN IN RUNOFF WATERS IN NORTHWESTERN SICHUAN OF SOUTHWESTERN CHINA

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Abstract. In studying the hydro-ecological conditions in northwestern Sichuan of southwestern China, an area at the edge of the Qinghai-Tibet Plateau, stream waters were extensively sampled and analyzed for the stable hydrogen and oxygen isotope ratios (δ^2H and $\delta^{18}O$) which are often excellent indicators for a water body genesis and evolvement. The heavy isotopes of hydrogen and oxygen were found to be, in general, gradually depleted from east to west in the region; the phenomenon is attributed mainly to continentality based on the assumption that the isotopic signatures in the precipitations were preserved in the stream waters. The δ^2H and $\delta^{18}O$ data from the Yangtze River basin were found to fall well on the global meteoric water line (GMWL), indicative of the fact that the runoff waters in this basin were primarily of meteoric origin with a regional weak evaporation prevailing during our sampling time. However, the isotopic data from a relatively small part of the area in the north belonging to the Yellow River basin are characterized by the appreciably flatter slope of the local water isotope line and lower d-excess values, suggesting that the steam waters had undergone significant evaporation due to kinetic fractionations of isotopes in a more arid climate. The stable isotope data were also applied in calculating water mixing for some selected rivers. This study represents a snapshot study of the area's hydro-ecology; more systematic studies are required in better understanding the seasonal hydrological variations.

Keywords: *meteoric water, hydro-ecology, δ^2H and $\delta^{18}O$, three-river headwaters (TRH), southwestern China*

Introduction

The hydro-ecological conditions of the Qinghai-Tibet Plateau are of critical importance to several Asian countries including a large part of China. The three-river headwaters (TRH) region, located in southern Qinghai province (31°39′–36°16′N, 89°24′–102°23′E), is known as China's water tower where China's three largest rivers (among the world's largest ones) originate. The Big Three, i.e., the Yangtze River, the Yellow River and the Lancang River, obtain, respectively, 25%, 50% and 15% of their water from the TRH region (Zhao et al., 2010). Because of the fragile primordial ecosystem and due to the increased anthropogenic activities during the past several decades, the eco-environmental problems in the region such as grassland degradation, wetland shrinkage and soil erosion started to accelerate since the 1980's and presently serious and immediate attentions are called for (Li et al., 2012; Gao et al., 2013). Li et al. (2012) found that the water loss due to evapotranspiration in the TRH region

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increased 11.6 mm annually between 1980 and 2000, meanwhile exhibiting an accelerating trend. It is therefore important to understand the hydro-ecological system of the region in order to conserve and restore its environmental conditions. However, this vast area is generally not easily accessible due to high altitude, harsh climate, extreme remoteness and lack of roads.

The northwestern Sichuan area studied in this work, with gradually decreasing elevations southeasterly, forms the easternmost part of the Qinghai-Tibet Plateau. This area can be divided into two sub-regions: the Northwestern Sichuan Plateau and the Southwestern Sichuan Mountainous Terrain (*Figure 1*). The former is characterized by vast and hummocky plateaus with high elevations (3,500 to 4,000 m or higher); the latter consists of numerous high mountain ranges separated by deep valleys with elevation differentials as much as 3,000 m, known generically as the Hengduan Mountains. The river systems and wetlands in the region are intrinsically connected with those in the upper part of the TRH region. These wetlands provide many important ecological services such as lessening the greenhouse effect by taking up and storing carbon, and are invaluable environmental and ecologic resources (Avis et al., 2011). The hydro-ecological condition of this region is an important integral part of the TRH regional ecology; a thorough understanding of this region may hold the key for the entire TRH region. These wetlands have experienced significant degradation over the past 4 decades and have shrunk by some 29%, and the wetland ecosystems are increasingly fragmented at an accelerated pace (Li et al., 2012; Qi and Li, 2007; Wang et al., 2007) mainly due to the increased anthropogenic activities in the region (Li et al., 2012). In order to stabilize and possibly reverse the ecological conditions of the region, the regional hydrology including the sources of the precipitations, the general water cycle processes of the drainage basins must be understood.

The stable isotopes of oxygen (^{18}O) and hydrogen (^{2}H) can be accurately determined (usually as isotope ratios) with modern technologies (Richelle et al., 2004; Bowen et al., 2005a; Paul et al., 2007), and have become some of the most widely used tracers in drainage basin hydrological studies (Clark and Fritz, 1997; Kendall and McDonnell, 1998; Buttle and McDonnell, 2004). In particular, stable isotope fractionation during precipitation processes are well understood, both ^{18}O and ^{2}H in precipitations vary spatially and temporally depending on the moisture sources and the environmental conditions (e.g., relative humidity and temperature) during cloud formation and rainfall. The isotope ratios in precipitations, their seasonal variations at a given location, and their distribution over a region often reflect the relevant meteorological and geographical conditions such as local temperature and temperature variation, distance from the coast (continentality), latitude and altitude, amount of precipitation and duration (Clark and Fritz, 1997).

Due to their spatial and temporal variability in meteoric waters, these isotope ratios ($\delta^{2}H$ and $\delta^{18}O$ values) have been used to identify sources of water such as atmospheric moisture (Liu et al., 2010; Sjostrom and Welker, 2009; Vachon et al., 2010; Welker, 2000), groundwater recharge (Abbott et al., 2000; Blasch and Bryson, 2007; Adomako

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et al., 2010), and plant water uptake (Brooks et al., 2010). In this work, the surface stream waters in the northwestern Sichuan region, an area covering part of both the Yangtze River and the Yellow River drainage basins (divided by the Chazhengliangzi Mountain in Hongyuan, Sichuan) were extensively sampled and analyzed for δ^2H and δ^{18}O. Most rivers in the region including the Minjiang, Jialing, Fuhe and Dadu flow from north to south or from west to east to the Yangtze River (Yangtze River drainage basin), whereas the Heihe and Baihe rivers from south to north to the Yellow River (Yellow River drainage basin). The main objective of this study is to enhance our understanding of the regional hydrology of the study area through characterizing the isotopic variation patterns of the runoff waters and identifying the main influential factors including the geographical and meteorological parameters. As the TRH region is difficult to access and its hydrology study is still at a relatively early stage, the current work may help fill some of the gaps in the study of the hydrological-ecological conditions of the entire TRH region.

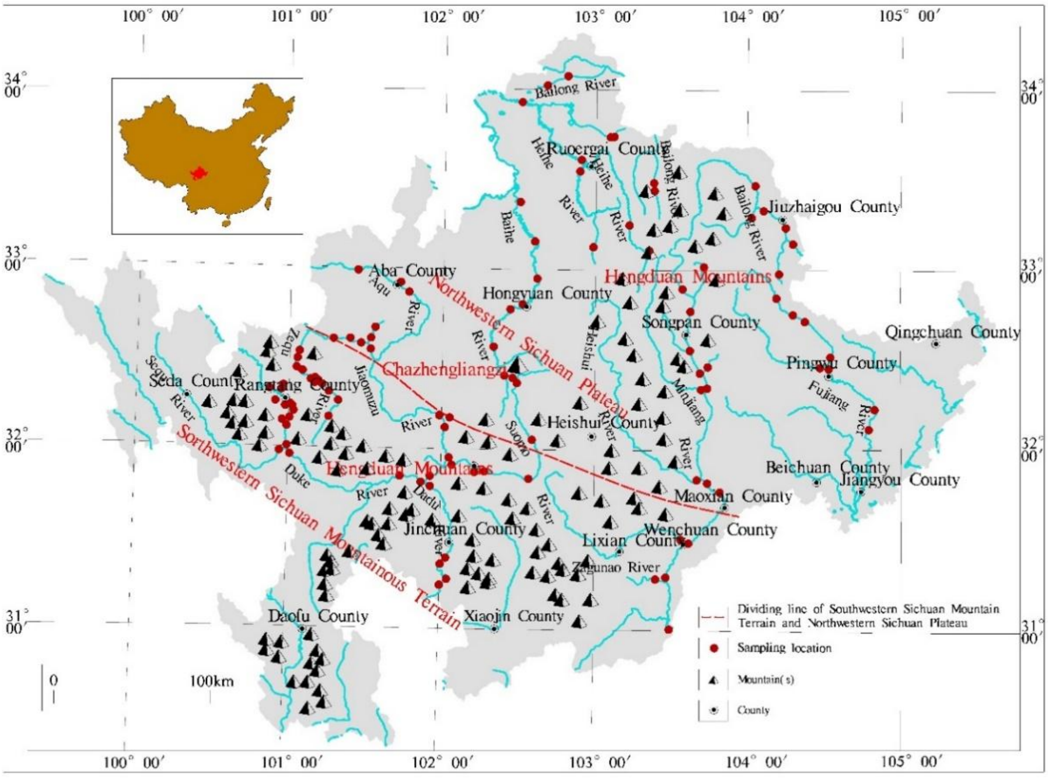

Figure 1. *Map of the main rivers in the study area with sample locations*

Materials and methods

Study area

The study area is at the edge of the Qinghai-Tibet Plateau and is generally known as the Northwestern Sichuan Plateau. Water samples were taken from locations in 28 counties: 13 alpine counties including Rangtang, Aba, Songpan, Ruoergai and

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Hongyuan in Aba Prefecture, and 18 mountainous counties including Seda in Ganzi Prefecture, Wangcang in Guangyuan City, Hanyuan in Ya'an City, and Pingwu in Mianyang City, covering a total area of 149,000 km^2. In total 86 stream water samples were collected for δ^2H and $\delta^{18}O$ analysis from 7 river systems (*Table 1*). Owning to logistic difficulties, the sample campaign had to be conducted in two consecutive summers of 2010 and 2011. Approximately half of the samples were taken in July of 2010 and half in July and August of 2011. The climatic conditions of the sampling area from June to August are summarized and compared in *Table 2* for 2010 and 2011 (data were downloaded from Tutiempo Network website, http://en.tutiempo.net/). From *Table 2,* it is clear that the climatic conditions of the area were closely similar between 2010 and 2011 during the sampling months. It is noted that August 2011 appeared to be drier than August 2010; however, this difference would not impact our discussions because the dry season essentially occurred after our sampling campaign.

Table 1. *Number of river water samples taken from various river catchments*

River Catchment	No. of Samples
Fujiang	8
Baishui	6
Heihe	7
Bailong	4
Minjiang	16
Duke	13
Zequ	8
Baihe	6
Suomo	5
Jiaomuzu	13
Sum	86

Table 2. *Comparison of summer climatic conditions between 2010 and 2011at selected locations in the study area*

	Average temperature (˚C)		Precipitation (mm)		Number of rainy days	
	2010	**2011**	**2010**	**2011**	**2010**	**2011**
Songpan, 32.65N 103.56E, Elevation 2852 m						
June	12.9	14.1	125.2	113.27	23	21
July	16.9	15.0	101.1	120.65	20	19
August	16.4	15.1	118.39	39.36	17	12
Seda, 32.28N 100.33E, Elevation 3896 m						
June	9.5	9.6	153.67	189.22	25	22
July	12.4	10.2	65.78	197.63	16	25
August	10.8	9.9	99	62.98	19	12
Daofu, 30.98N 101.11E, Elevation 2959 m						
June	15.4	15.8	157.21	118.12	22	21
July	17.9	15.9	111.76	121.66	17	20
August	16.9	16.2	108.2	63.25	17	9

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Ganzi, 31.61N 100E, Elevation 3394 m						
June	13.5	13.7	157.99	121.91	26	23
July	16.2	14	71.65	138.69	19	23
August	15.3	13.8	89.9	71.36	21	13
Ma'erkang, 31.9N 102.23E, Elevation 2666 m						
June	15.1	15.4	155.96	169.17	27	21
July	17.7	15.5	180.6	157.22	24	22
August	16.9	16.5	145.31	42.42	24	11

Data from: http://en.tutiempo.net/

Methods

The sampling locations are listed in *Table 3* and shown on the map in *Figure 1*. We have exercised rigorous and systematic quality assurance measures in the field at various levels to ensure the samples were taken and labeled appropriately. We conducted the sample taking following strictly the procedures set out in the Quality Assurance Manual for Environmental Water Monitoring (Zhang, 1994). The water samples were taken at a depth of a few centimeters below the water surface to ensure that the river water was fully mixed to avoid any potential surface isotope fractionation effect due to evaporation. When sampling from a water body where two streams met, samples were taken only in the fully mixed zone, 200 m or further downstream from the meeting point. During sampling, observations of water quality, river flow rate, vegetation on both banks, rock composition and geological features were also made. More details on sampling was described elsewhere (Shi et al., 2016).

Table 3. *Summary of measured sample isotopic data (δ^2H and $\delta^{18}O$ values), associated location, and other information*

Catch.	Location	Elev(m)	Temp(°C)	δ^2H	δ^{18}O	d-excess
Fujiang River	5 km north of Xiangyanzhen	648	25.0	-65.5	-9.74	12.4
	2 km south of Pingwubazi	716	25.0	-66.8	-9.97	13.0
	3 km north of Pingwu	744	17.1	-64.7	-9.84	14.1
	1.5 km from Dapingli near Huanglong	899	17.3	-64.7	-9.83	13.9
	2 km from Liangheshui (Jiuzhaigou direction)	890	18.2	-65.3	-9.76	12.7
	500 m downstream from Hua'neng Hydro Station	1121	12.6	-70.4	-10.54	13.9
	Near Shuiniujia, Baima Township	2110	16.6	-65.8	-9.68	11.6
	Yangdong River Divide downstream near source	2387	13.2	-67.3	-10.07	13.3
Baishui River	At Wujiao, Jiuzhaigou	1598	16.4	-66.7	-10.18	14.8
	2 km from Shuanghe (Wenxian direction)	1430	17.1	-77.0	-10.96	10.7
	2.5 km south of Jiuzhaigou	1400	17.6	-75.8	-11.07	12.8

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	1 km upstream Baihe Township (Heihe)	1562	14.5	-76.5	-11.16	12.8
	Under Yanlicun Bridge, Baihe Township	1789	13.8	-76.7	-11.19	12.8
	5 km upstream from Jiuzhaigou mouth	2179	11.3	-78.7	-11.49	13.2
Heihe River	10 km north of Songpan and Ruoergai border	3693	19.3	-83.9	-11.55	8.5
	25 km southeast of Rouergai	3522	20.0	-83.7	-11.32	6.8
	1 km west of Heihe divisional ranch	3429	19.9	-80.8	-10.36	2.1
	Creek, front of Axi Government Building	3493	19.5	-83.6	-11.35	7.2
	4 km northwest of Ruoergai	3437	18.0	-81.7	-10.23	0.2
	Heihe divisional ranch	3442	21.2	-80.5	-10.03	-0.3
	Maiwaxiang, Hongyuan	3555	21.6	-89.0	-12.67	12.4
Bailong River	2 km downstream Jiangza township	3019	14.8	-79.7	-11.21	10.0
	Upstream Hongxing township, near source of Bailong River	3186	14.1	-81.0	-11.15	8.2
	In Baozuo Township yard	3022	14.1	-83.0	-11.78	11.3
	500 m downstream from Baozuo Township yard	3009	20.0	-83.8	-11.85	11.0
Minjiang River	8-10 km downstream from Minjiang source	3316	13.6	-84.7	-12.16	12.5
	5 km from Chuanzhusi (Ruoergai direction)	3021	14.8	-83.3	-11.79	11.1
	8 km downstream from Chuanzhusi	2921	15.8	-86.2	-12.25	11.8
	Munigou mouth	2706	13.0	-85.6	-12.16	11.7
	Under bridge near Daxing'gou mouth	2581	14.5	-89.1	-12.65	12.1
	Minjiang mainstream	2568	17.1	-85.3	-11.99	10.6
	Minjiang mainstream	2491	17.1	-86.5	-12.33	12.2
	Mouth of Xiaohegou	2487	19.6	-81.8	-11.56	10.8
	Downstream from Heishuihe	1650	18.4	-87.1	-12.30	11.3
	Minjiang mainstream, downstream of hydro station	1653	17.6	-81.7	-11.69	11.8
	Minjiang mainstream, under Juanmenxiang Bridge	1649	20.5	-84.5	-12.11	12.4
	Minjiang mainstream, behind Zhongxing	1353	19.8	-83.4	-11.90	11.8
	Upstream of Zagu'nao River Hydro Station	1384	16.6	-90.6	-12.69	10.9
	Minjiang mainstream at Yinxing township	991	18.7	-85.0	-11.98	10.8
	Shaotang River tributary	898	24.5	-83.7	-12.07	12.9

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	Minjiang mainstream	711	21.8	-83.1	-11.96	12.6
Duke River	Gangmudarewo Village	3298	13.4	-98.3	-14.02	13.9
	Rangkegare Village	3253	15.3	-98.7	-14.01	13.4
	Gangmudake Village	3205	12.9	-97.7	-13.76	12.4
	Rangtangdamuda Village	3277	13.0	-98.8	-13.80	11.6
	Sequ River at Heiqiao	3008	15.5	-107.5	-14.87	11.4
	Heiqiao, Rangtang	3007	14.0	-99.1	-13.97	12.6
	Ergewu Village, Heiqiao	3009	14.8	-101.2	-14.22	12.6
	Guanyinqiao, Jinchuan	2078	14.0	-103.3	-14.46	12.4
	Baiwan, Ma’erkang	2300	17.7	-101.0	-14.41	14.3
	Gangmudarewo Village	3382	9.1	-99.8	-13.85	11.0
	Gangmudarewo Village	3341	12.3	-100.4	-14.11	12.6
	Rangkegare Village	3264	9.6	-107.4	-14.71	10.3
	Gangmuda’angke Village	3193	12.0	-106.3	-14.86	12.6
Zequ River	Xumuda Village, Shangrangtang township	3480	13.0	-100.7	-13.96	11.0
	Upper Rangtang	2869	13.1	-100.9	-14.09	11.8
	Middle Rangtang	3489	13.7	-101.1	-14.10	11.8
	Yigenmenduo Village, Middle Rangtang	3412	13.4	-101.8	-14.21	12.0
	Gaduo, Rangtang	3480	14.9	-100.1	-14.23	13.8
	South Muda, Rangtang	3362	15.9	-100.3	-13.98	11.6
	Rongmuda Village	3292	18.5	-100.5	-14.32	14.1
	Yigenmenduo Village	3411	13.2	-101.5	-13.44	6.0
Baihe River	Xigou, Hongyuan	3635	11.0	-94.1	-13.29	12.2
	Anqu, Hongyuan	3519	13.5	-92.5	-13.46	15.2
	Hongyuan	3491	15.3	-96.8	-13.55	11.6
	Baihe at Tangke	3463	17.3	-87.6	-11.81	6.9
	5 km upstream Waqie	3462	16.4	-88.8	-12.16	8.5
	Baihe, upstream Amu Township	3477	17.8	-87.7	-11.90	7.4
	Rangkou, Hongyuan	3574	11.5	-96.3	-13.39	10.9
	Laomaokang Village	3225	10.8	-95.9	-13.21	9.8
	Suomo, Ma’erkang	2530	10.8	-100.1	-13.71	9.6
	Ma’erkang	2645	11.4	-96.5	-13.98	15.3
	Songgang, Ma’erkang	2495	13.3	-96.1	-13.83	14.5
Jiaomuzu River	Jia’erduo (Aqu River)	3430	17.5	-84.1	-11.93	11.4
	Anjiang (Aqu River)	3248	17.8	-87.1	-12.38	11.9
	Kehe Village (Aqu River)	3070	15.4	-91.3	-12.73	10.5
	Kuasha, Aba	2985	15.4	-92.9	-12.91	10.4
	Kuasha (Aqe River)	2985	13.5	-85.7	-12.20	12.0
	Kuasha (Ma’erqu River)	2985	15.0	-86.2	-12.38	12.9
	Gabo, Ma’erkang	2536	15.4	-92.1	-13.01	12.0

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	Gabo	2496	15.3	-96.6	-13.84	14.1
	Jiaomuzu, Ma'erkang	2500	15.8	-92.0	-13.09	12.7
	Baiwan, Ma'erkan	2344	16.1	-92.0	-13.11	12.9
	Shuangjiangkou, Ma'erkang	2318	17.2	-99.5	-13.82	11.1
	Kehe Village	3064	20.0	-100.1	-13.83	10.6
	Gabo	2469	13.1	-96.7	-13.95	15.0

Notes：Catch. - Catchment; Elev - Elevation; Temp - Temperature

Hydrogen and oxygen isotopes (δ^2H and $\delta^{18}O$ values) were determined by the Sichuan Provincial Key Isotope Laboratory at the Chengdu University of Technology, using high temperature conversion elemental analysis – isotopic ratio mass spectrometry (TC/EA-IRMS) with the instruments supplied by Thermo Fisher (TM Flash 2000 coupled with a mass spectrometer, MAT-253). The accuracy of the isotopic ratio analysis relative to the Vienna-Standard Mean Ocean Water (VSMOW) was determined to be ±0.2‰ and ±2.0‰ for $\delta^{18}O$ and δ^2H, respectively. Water samples were analyzed without pre-treatment. For more details about the analytical procedure used, the reader is referred to Burgoyne and Hayes (1998), Hoefs (2009), and Richelle et al. (2004).

Results and discussion

$\delta^{18}O$, δ^2H, d-excess and local meteoric water line (LMWL)

The measured stable hydrogen and oxygen isotope δ-values are presented in *Table 3* together with the associated information of sample locations and the relevant river catchments/drainage basins. Because altitude and temperature are two of the most important parameters governing isotopic fractionations in precipitation (see discussions below), these two parameters were therefore also measured and are included in *Table 3*. Since the climatic conditions in 2010 and 2011 were very similar during the sampling times (*Table 2*), the measured isotopic data were analyzed and discussed without distinction of the sampling year. The parameter d-excess can convey additional information about the water bodies studied, therefore the derived d-values are also tabulated in *Table 3*.

The measured $\delta^{18}O$ and δ^2H values range from, respectively, -14.87‰ to -9.68‰ and -107.5‰ to -64.7‰ for the water samples taken from the various river systems in the study area. When the data are plotted in a δ^2H–$\delta^{18}O$ diagram as shown in *Figure 2,* the data points align well with the GMWL as defined by Craig (1961), suggesting that the area's stream water was likely of meteoric origin primarily. Recently, Liu et al. (2014) studied and reported the isotopic compositions of precipitations in China based on the data collected from 29 stations including two within the present study area: 1. Maoxian (MX), elevation 1,826 m, located near the southeast border of the study area, and 2. Gonggashan (GG), elevation 2,950 m, located at the southwest border of the study area.

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The reported δ^2H values are from -83.3 to 8.5 with a mean of -49.5 for MX, from -147.8 to 1.4 with a mean of -76.5 for GG; and $\delta^{18}O$ from -11.98 to 0.31 with a mean of -7.77 for MX, from -19.50 to -2.35 with a mean of -10.82 for GG. These data compare very well with our data of samples from the same general areas (see *Figure 2* and *Table 2* for data from Fujiang River and Duke River, respectively) considering that the data by Liu et al. (2014) are monthly precipitation-weighted averages while our data are one-time values. Therefore, it is assumed in this work that the samples taken from the streams represent the local precipitations in terms of isotopic compositions. Our data span relatively narrow ranges when compared to the China national data of $\delta^{18}O$ (-29.47 to -2.75 ‰) and δ^2H (-229.6 to 45.4 ‰) (Liu et al., 2014). This is, perhaps, expected due to the fact that the area is located in the Asian monsoon region and our sampling times coincided with the monsoon prime times (Liu et al., 2014). The area was receiving rainfalls almost daily during our sampling times with the total precipitations of 100-200 mm/month. As the sampling was conducted during the warmest time of a year, the melting of alpine snow and ice was also expected at the peak time. There is usually no isotopic fractionation during snowfalls as the solid phase does not exchange isotopes with the atmospheric moisture; and snowpacks tend to conserve the isotopic composition of the cloud moisture with an elevated d-excess value (Gat et al., 2001). The value of d-excess varies locally and was shown to correlate with the physical conditions such as relative humidity, air temperature and sea surface temperature of the oceanic source area of the precipitation moisture (Merlivat and Jouzel, 1979). Thus the waters would be expected to to be more enriched with heavy isotopes and with an elevated d-excess if the streams were heavily recharged by meltwaters, and this is not the case in this work with Aqu River as possibly an exception (*Table 3*). Because of the heavy and frequent rainfalls, the ground water contribution to the streams is also considered to be insignificant. The “catchment isotope effect” (Rozanski et al., 2001) in the region during the sampling times was such that the isotopes of the steam waters may be considered having essentially the same composition as those in the precipitation waters.

As noted earlier that the isotopic ratios (δ^2H and $\delta^{18}O$ values) are distributed quite evenly along the GMWL in *Figure 2* (when the data from the Yellow River basin are excluded, see below for details). This may indicate that, as the stream waters were primarily of meteoric origin, the rain moisture may originate primarily from a single remote location and rainfalls formed along the way with different distances of moisture transport. Recently, Wang et al. (2016) used the entropy analysis method of stable isotopes in precipitation to study the monsoon systems in China, found that in southern China, the water vapor is mainly supplied by the Indian monsoon. “one stream of the Indian monsoon (I1) passes eastward from the southwestern border of China and then turns to the northeast of the south central China before reaching eastern China, where its intensity weakens after producing heavy rainfalls.” However, the authors also recognized the impeding effect of the Himalayas that impedes the movement of the Indian monsoon from the south to north and the movement of the winter monsoon from the north to south (Wang et al., 2016).

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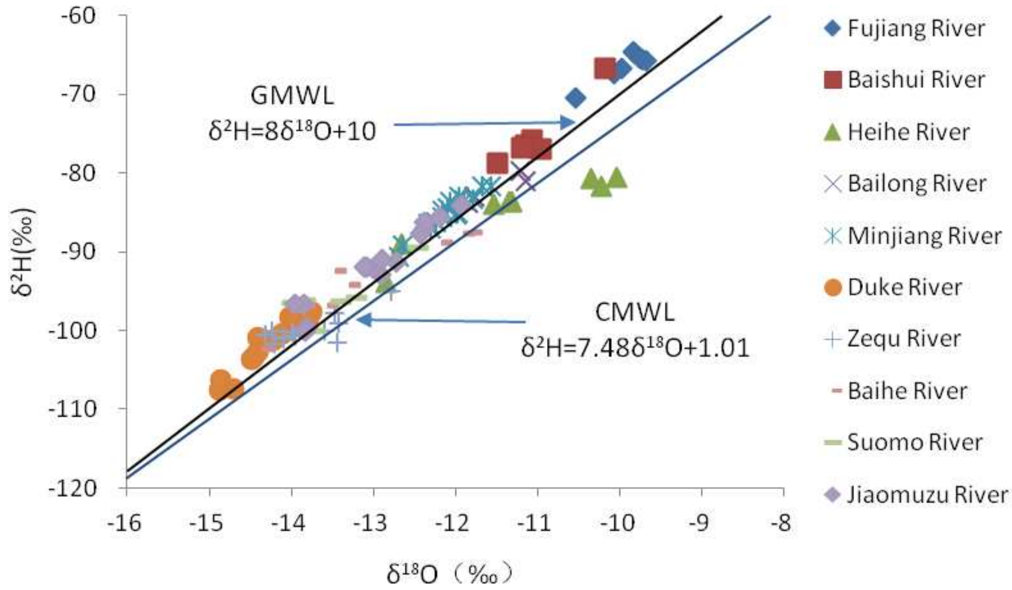

Figure 2. *Diagram of hydrogen and oxygen isotopic ratios (δ^2H vs. $\delta^{18}O$) for all water samples analyzed in this study. Note that the upper line represents the global meteoric water line (GMWL), and the lower line represents the China meteoric water line (CMWL)*

These observations are also supported by the d-excess data. In the study area the d-values span a relatively large range from -0.25 to 15.33 with a mean of 11.30 (*Table 3*), which is close to the global average (Rozanski et al., 1993). In examining the d-values, no apparent anomalies nor any reasonable patterns (e.g. decreasing values when further away from alpine glaciers) could be found, suggesting a predominantly well-mixed, relatively homogeneous source for the river waters, and contributions from the melting of alpine snow and ice were likely insignificant. Lower d-values relative to the surrounding areas were found in the Heishui County region (*Figure 3*); this may be attributed to high evaporation in the region where abundant sunshine is available throughout the year and the landscape is characterized by interweaved high mountain ranges and deep valleys (elevation differential as much as 1,000–2,000 m). This unique combination of climatic and geographic conditions gave result to widely uneven precipitations and large temperature swings between day and night in the Heishui region. However, the rather low d-values may not be fully counted for by the evaporation effect; it is suspected that some water component previously affected by evaporation and stored in the riparian zone might have been pushed into the streams by the newly fallen precipitation through infiltration and hyporheic flow (National Research Council, 2002). The lower d-values found in the Heihe River and Beihe River area will be further discussed in a later section.

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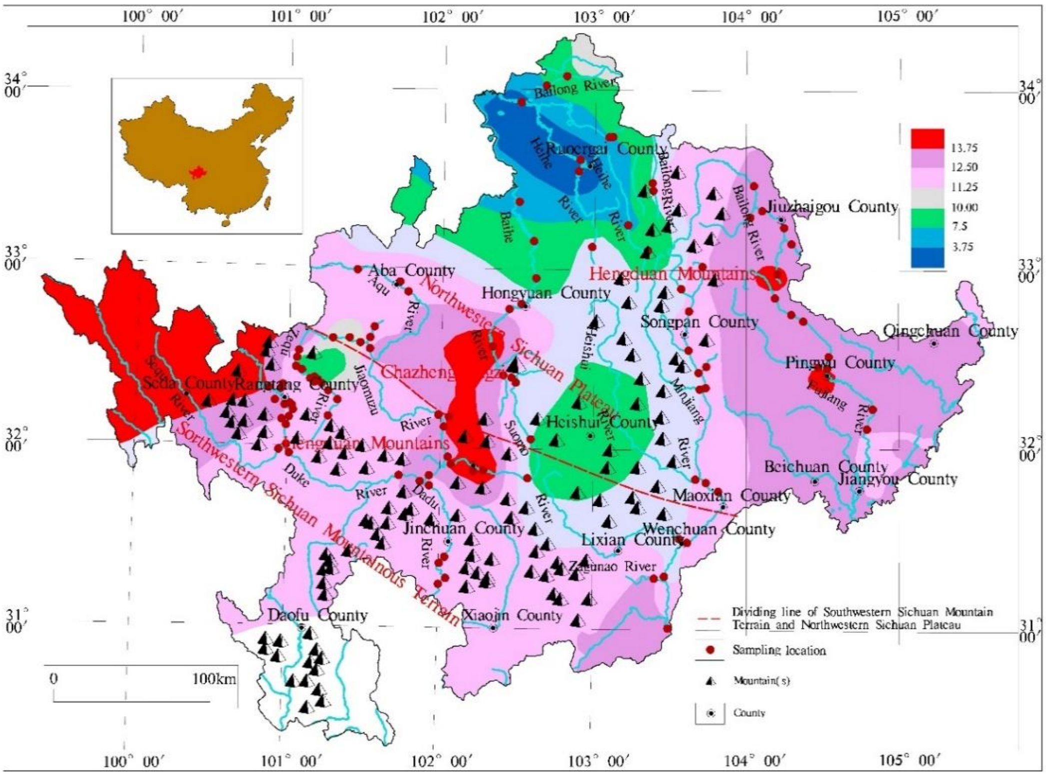

__Figure 3.__ Spatial distribution of d-excess in the study area

Effects of temperature, altitude and continentality

The stable isotope ratios δ^2H and $\delta^{18}O$ are shown in *Figures 4* and *5* as mapped contours through interpolation. In examining the contours in both figures, it is noted that there is a general trend: both ^2H and ^{18}O become progressively more depleted from east to west across the region. The study area is located inland at the west margin of the south China (SC) region and near the Tibet (TP) and north China (NC) regions according to Liu et al. (2014). The precipitation moisture of this area has a complex origin and is composed of components from the South China Sea, the Pacific and Indian Oceans, and local evaporation and circulation. This complex situation may be visually appreciated by the back-trajectories of the air masses on June 30 and July 4, 2010 and 2011 from two selected locations near the center of the studied area depicted in *Figure 6,* produced using the HYSPLIT transport and dispersion model (Stein et al., 2015; Rolph, 2016). In this region, the air mass has a general trend of undergoing progressive rainout of heavy isotopes during the pole-ward (latitude effect) and westward (continental effect) atmospheric transport (Liu et al., 2014). However, the southwesterly monsoon from the Indian Ocean may be effectively obstructed from reaching here by the Qinghai-Tibet Plateau, this is supported by studies such as Liu et al. (2007, 2014). It is postulated that the moisture for precipitation in this region originated primarily from the Pacific Ocean and the South China Sea during a period shortly prior to the sample campaigns. That is, the moisture air masses traveled mainly from east to west, isotopically enriched rain

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formed and fell from diminishing moisture mass and the residual moisture became progressively depleted in heavy isotopes with respect to earlier rains from the same cloud. This westerly continental effect explains well the observed isotope ratio contours seen in *Figures 4* and *5,* where no apparent pole-ward isotopic fractionation pattern is observed which is perhaps due to the ineffectiveness of the southwesterly monsoon in penetrating the Qinghai-Tibet Plateau (Liu et al., 2007, 2012).

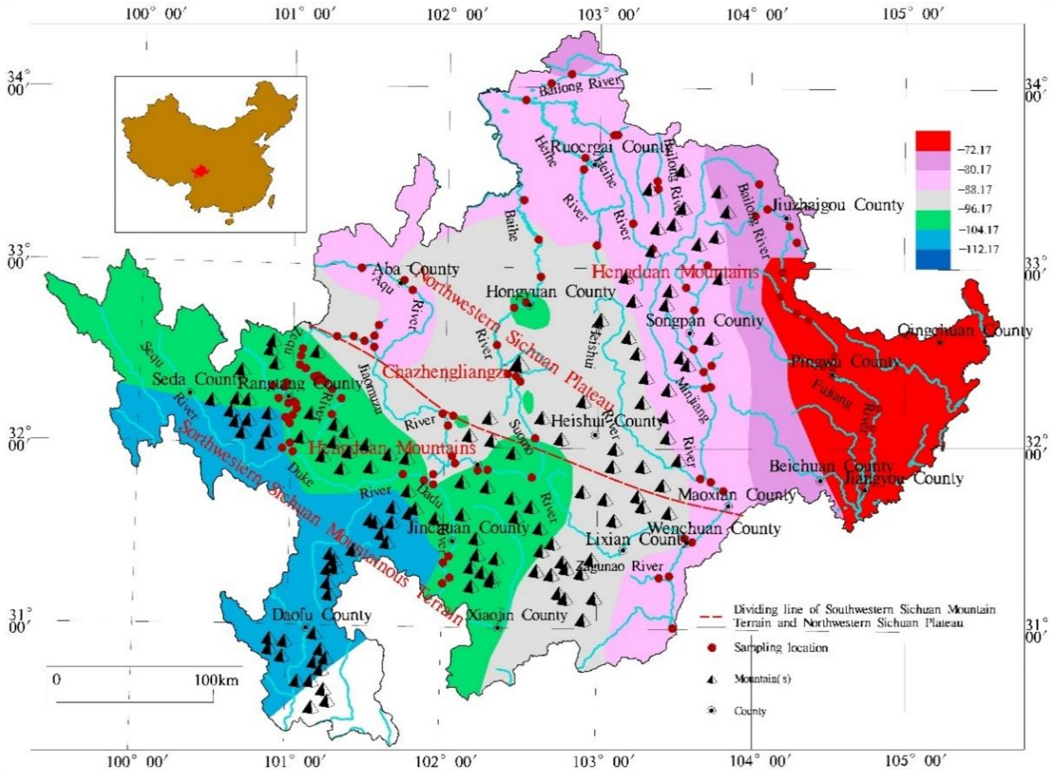

Figure 4. *Spatial zonation of δ²H in the study area*

This noted general trend of isotopic composition change from east to west due to continental effect is fundamentally governed by temperature change. With decreasing temperature, rainfalls becomes more depleted in the heavier isotopes, ^{2}H and ^{18}O (^{2}H and ^{18}O are linearly correlated). Similar effects have been shown with increasing elevation and increased distance from the equator, both of which correspond to lower temperatures (Bowen et al., 2005b; Terzer et al., 2013). Our statistical analysis shows that both $\delta^{2}H$ and $\delta^{18}O$ are positively correlated with temperature, the Pearson product-moment correlation coefficients 0.492 and 0.517 for $\delta^{2}H$-temperature and $\delta^{18}O$-temperature, respectively, were obtained. Some early work (e.g., Zhang and Wu, 2007) suggests that the correlation between isotopic fractionation and temperature tends to be stronger when the temperature is lower.

Though the general zonation of $\delta^{2}H$ and $\delta^{18}O$ in the region (*Figures 4* and *5*) is believed to be governed by continentality, with increasing altitude the elevation and

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other effects (e.g. amount effect) are superimposed on the continental effect and might become predominant resulting in a local enrichment of the heavier isotopes. This may be observed in *Figure 5* where both ^{2}H and ^{18}O are noticeably more depleted near Hongyuan presumably due to high altitude. Notwithstanding the altitude effect, the other factors including the amount effect (Dansgaard, 1964) and anthropogenic effect may also have played a role that should be fully investigated. For example, the east part of the study area is more densely populated and economically more developed; the increased anthropogenic activities including large amount of agricultural consumption of surface waters might have cut down the water runoff volumes and augmented natural evaporation and transpiration in the area, resulting in a more arid-like climate which in turn resulted in heavier isotopes relatively enriched (*Figures 4* and *5*).

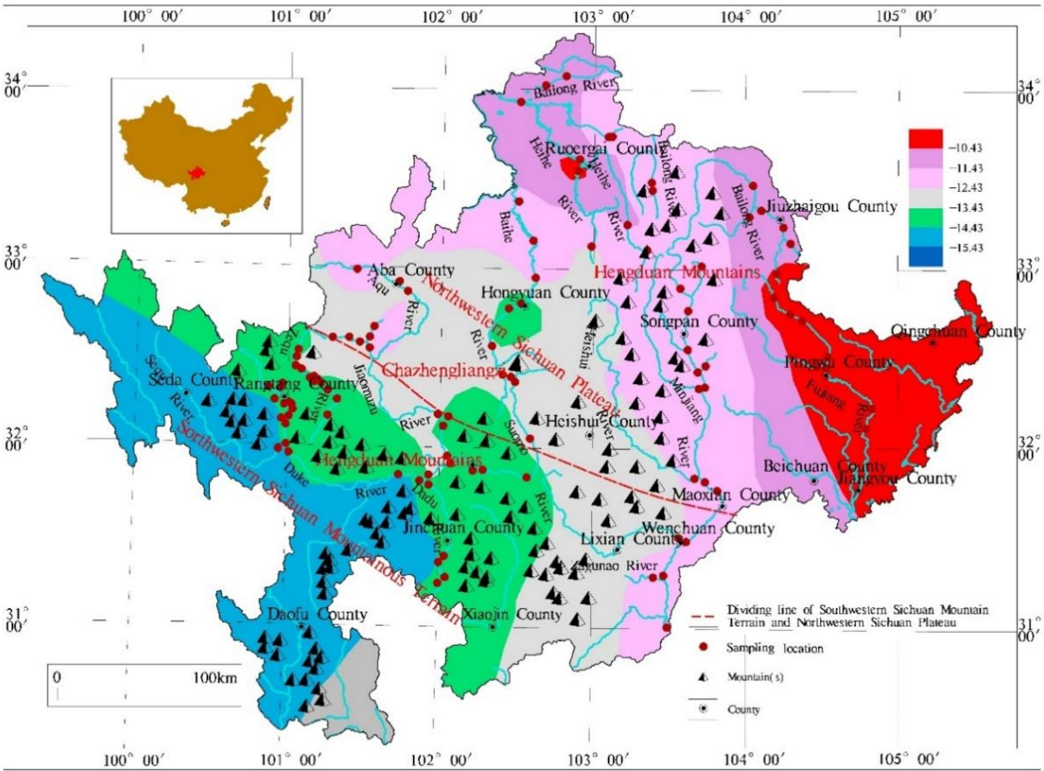

Figure 5. *Spatial zonation of* $\delta^{18}O$ *in the study area*

Long-term data sets are necessary to fully understand the processes of water cycles because seasonal and climate changes can be significant (Stumpp et al., 2014). For this reason, the results reported in this work may be regarded as a snapshot of the area's water cycle processes at the wettest time of a year and much of the discussion should be taken in this context. As seasonal variations are expected to be greater for rivers that are mainly recharged by surface runoff from recent precipitations (Rozanski et al., 2001) such as the rivers in this study, more systematic sampling and investigations are necessary to fully understand the area's runoff isotope hydrology.

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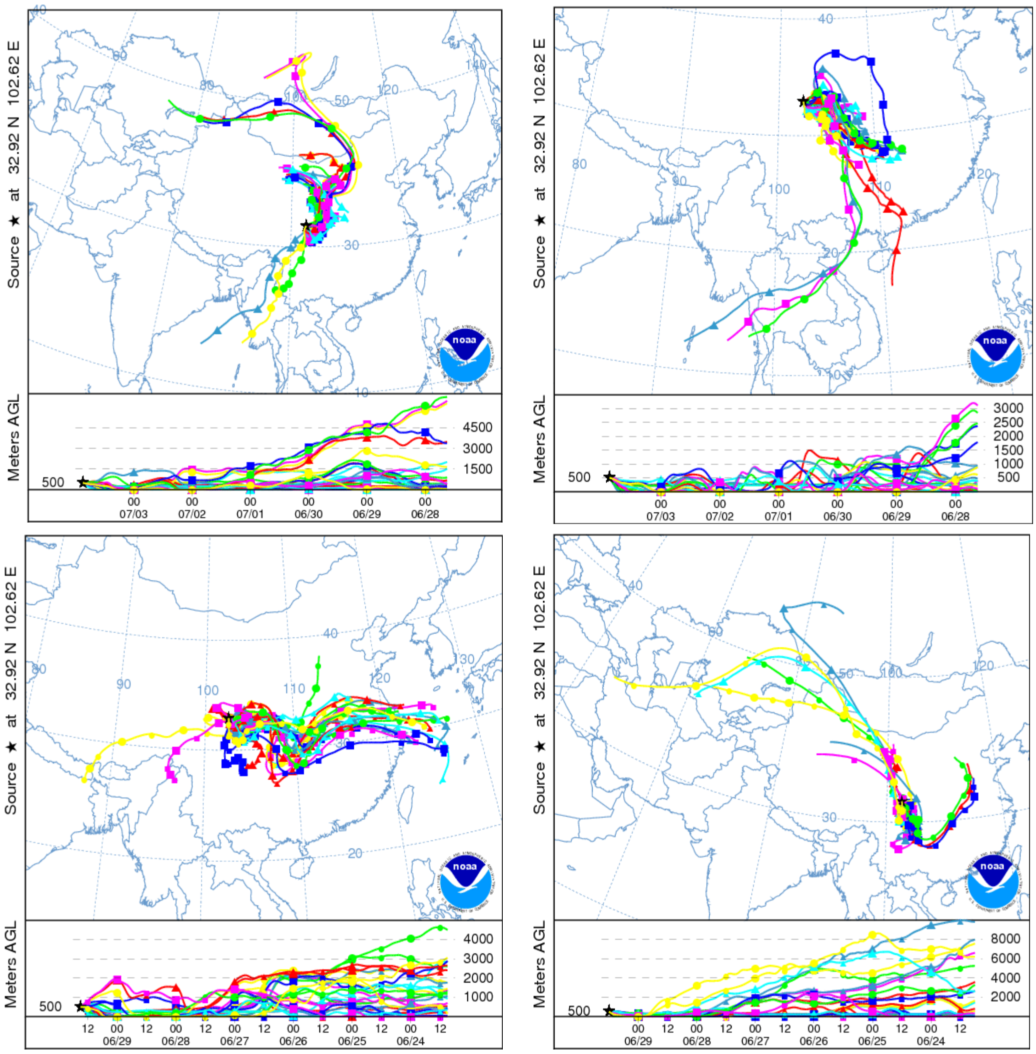

Figure 6. *Week-long back trajectories of air masses on June 30 (bottom) and July 4 (top), 2010 (left) and 2011 (right) at 2 selected locations near the center of the study area*

Isotope distinction between the drainage basins of Yangtze River and Yellow River

The meteoric water line specific to a certain location is controlled by local climatic factors including the oceanic moisture source and secondary processes affecting the isotopic composition of water vapor such as evaporation and re-circulation of moisture from large water bodies (Clark and Fritz, 1997). These factors together define a particular local meteoric water line (LMWL). A closer examination of *Figure 2* reveals that some data points deviate appreciably from the GMWL which otherwise represents the stable hydrogen and oxygen isotope ratio data quite well. However, when only the data from the Yangtze River basin are plotted in *Figure 7,* a single LMWL is obtained: $\delta^2H=8.06\delta^{18}O+12.69$ with $R^2 = 0.98$, which is almost identical to the GMWL as revised by Rozanski et al. (1993): $\delta^2H=8.17\delta^{18}O+10.35$, or by Gat et al. (2001): $\delta^2H=7.8973^{18}O+9.0133$. This is indicative of the fact that heavy and frequent rainfalls prior to our sampling in the region had rendered the stream waters resemble the

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rainwaters so closely, and the streams in the Yangtze River drainage basin were recharged and maintained mainly by the rainfalls and the surface runoff. The lower the relative humidity is, the faster the evaporation rate would be and the greater the kinetic fractionation becomes. Humidity affects hydrogen and oxygen fractionations differently such that the slope of the evaporation line would vary due to changes in relative humidity. At very low relative humidity (< 25%), the slope of the evaporation line would be close to 4; for moderate relative humidity (25% to 75%) the slope would be between 4 and 5; only for relative humidity above 95% would the slope approach 8, i.e., the slope of the meteoric water line (Clark and Fritz, 1997). Therefore, from *Figure 7* it is inferred that humid conditions likely prevailed with relative humidity above 95% much of the time in the region. For a comparison, Liu et al. (2014), based on 928 groups of precipitation data, reported a Chinese Meteoric Water Line (CMWL) as $\delta^2H=7.48\delta^{18}O+1.01$, as also shown in *Figure 7*. It appears that there is a fair amount of difference between our local water line (i.e., LMWL) and the CMWL; however, considering our data range is relatively very narrow when compared to the data used for deriving the CMWL (Liu et al., 2014), the difference is not that significant. Nevertheless, it is noted that our data are systematically located above the CMWL, which is reflected by the relatively big difference in the d-values (12.69 vs. 1.01) indicating that the source of vapor for the area's precipitation was likely unique, i.e., sufficiently different from the bulk from that the CMWL was derived.

As noted earlier, the rivers belonging to the Yangtze River basin have rather different characteristics of stable hydrogen and oxygen isotopes from west to east across the area (*Table 3*). In the west of the Northwestern Sichuan Plateau the rivers including the Duke River, the Zequ River, the Suomo River, and the Jiaomuzu River are relatively enriched in the light isotopes; an exception is the Aqu River, a tributary of the Jiaomuzu River, which is relatively enriched in heavy isotopes and its d-values are lower than the other rivers. In comparison, the rivers in the east of the Northwestern Sichuan Plateau including the Fujiang River, the Minjiang River, and the Baishui River are relatively enriched in heavy isotopes. As discussed above, the isotope ratios change from east to west likely due to the continental effect, whereas the Aqu River was probably also appreciably recharged by alpine lake waters which were collection of glacier meltwater. However, the hydrogen and oxygen isotope ratios of the Fujiang River and the Minjiang River also have a trend of increasing from north to south possibly due to the altitude effect as the elevations increase appreciably (by as much as 2,000 m) from north to south along the two rivers. Besides, these two rivers have been more significantly affected by anthropogenic activities that may have added additional impact to the hydrogen and oxygen isotope ratios. For example, the water samples taken from the lower reach of the Minjiang River are slightly enriched in heavy isotopes with δ^2H /$\delta^{18}O$ as -70.4/-10.54 and -81.7/-11.69 respectively from two locations (500 m downstream of the Yilixiang Hua'neng Hydro Station, and downstream after the Heishui River enters the Minjiang River), compared to the values of -90.6/-12.69 in its upper reach (upstream of the Zagu'nao River Hydro Station).

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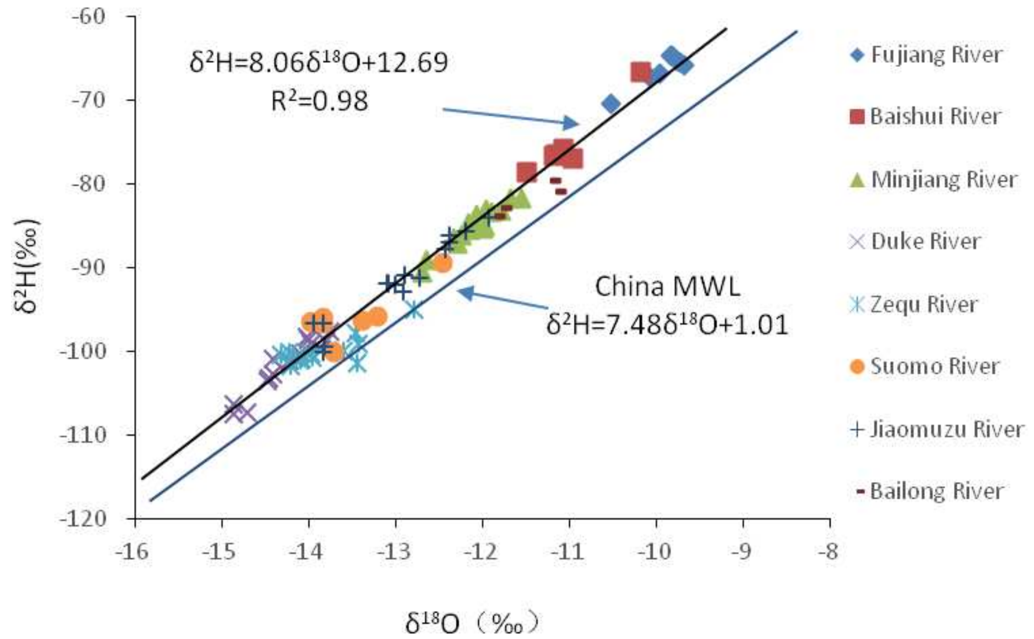

Figure 7. *δ^2H vs. $\delta^{18}O$ diagram for stream water samples collected from the Yangtze River basin*

The relatively small area in the northern part of the study area (*Figure 1*) including the Heihe River and the Baihe River catchments belongs to the Yellow River drainage basin. When the stable hydrogen and oxygen isotope ratios from this area only (i.e., the data points that deviate from the GMWL in *Figure 2*) were plotted as shown in *Figure 8*, a local evaporation line (LEL) was obtained: $\delta^2H=4.77\delta^{18}O–30.99$ with $R^2=0.93$, which differs appreciably from the GMWL (Rozanski et al., 1993; Hoefs, 2009), the CMWL (Liu et al., 2014), and the LMWL of the Yangtze River basin (*Figure 7*). The much smaller slope (4.77) is usually an indication of a more arid climate (i.e., lower relative humidity) (Gat, 1996).

The δ^2H and $\delta^{18}O$ values of water samples from the Yellow River basin range from -100.1 to -79.7 and from -13.98 to -10.03, respectively, varying over wider ranges (particularly the Heihe River) in comparison with those from the Yangtze River basin. In particular, the Heihe River and the Baihe River extend hundreds of kilometers from south to north before eventually flowing into the Yellow River, and their hydrogen and oxygen isotope compositions exhibit a general trend of increasingly more depleted in heavy isotopes. The d-excess data are presented as contoured lines in *Figure 3;* the d-values of water samples from this area are noticeably lower with a minimum of -0.25, also exhibiting a trend of decreasing from the upper reach to the lower reach of the rivers (i.e. form south to north). According to Gat (1996), in more arid areas evaporation from the falling rain droplets beneath the cloud base or from surface waters can result in the enrichment of the heavy isotopes in the remnant raindrops along the evaporation line, the resulting precipitation would show a smaller d-value

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than at the cloud base. Therefore, even though both this small area belonging to the Yellow River basin and the east part of the study area display some isotopic features in common (e.g., relatively less depleted in heavy isotopes and smaller d-values), the underlying governing factors are different. This area is at or near the north China (NC) region where $\delta^{18}O$ was shown to be closely related to the wind directions, relative humidity and vapor pressure; this is distinctively different from the case of the SC region where $\delta^{18}O$ was shown to depend on the remaining fraction of the vapor phase in an isothermal condensation process (Liu et al., 2014).

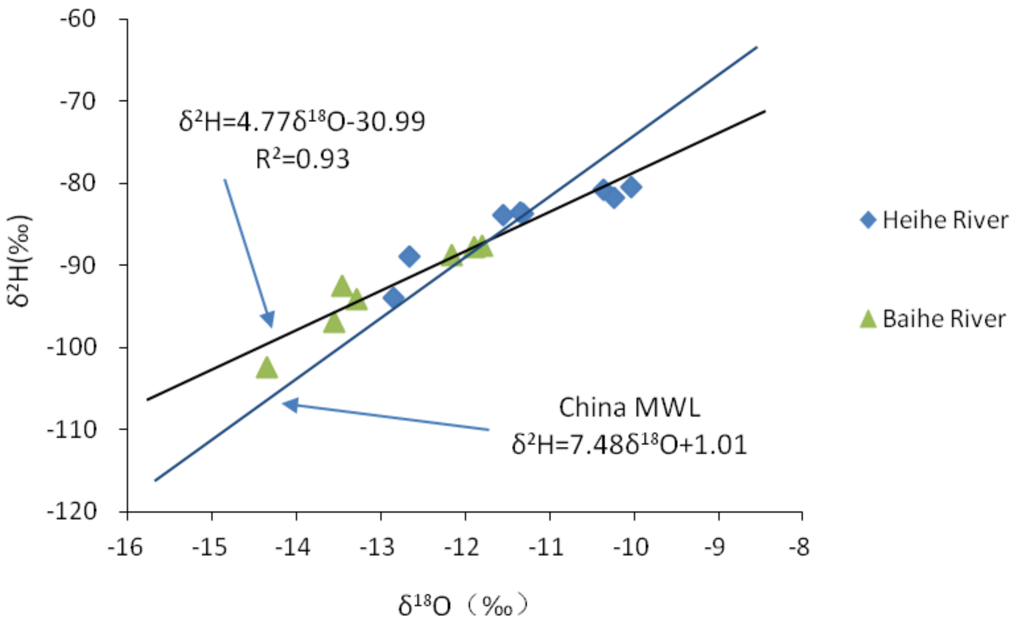

Figure 8. *δ^2H vs. $\delta^{18}O$ diagram for water samples collected from the Yellow River basin*

Mixing of some major rivers

When two or more tributaries merge to form a mainstream, the mixing ratio from each can be quantitatively calculated based on the isotopic mass balance principle. The fundamental equation (*Eq. 1*) can be written as:

$$r_T m_T = r_1 m_1 + r_2 m_2 + \cdots + r_n m_n \quad \text{(Eq. 1)}$$

where *r* is the fractional abundance of a stable isotope, *m* the molar quantity of the stable isotope, and the subscripts *T* and *n* denote, respectively, the merged mainstream and a tributary. When 2 tributaries merge to form a mainstream, the mass balance equation (*Eq. 2*) for ^{18}O can be written as:

$$r_{^{18}O} m_{^{18}O} = r_{^{18}O_1} m_{^{18}O_1} + r_{^{18}O_2} m_{^{18}O_2} \quad \text{(Eq. 2)}$$

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where (*Eq. 3*):

$$r_{^{18}O}=\frac{m_{^{18}O}}{m_{^{18}O}+m_{^{16}O}} \quad \text{(Eq. 3)}$$

For all practical purposes, r_{18_O} may be substituted by $\delta^{18}O$ and *Eq. 2* becomes (*Eq. 4*):

$$\delta^{18}Om_{^{18}O} = \delta^{18}O_1 m_{^{18}O1} + \delta^{18}O_2 m_{^{18}O2} \quad \text{(Eq. 4)}$$

Eq. 4 is not exact because of the approximation of the δ values for *r* values. The errors due to this approximation have been carefully evaluated and proven negligible for all practical applications (Fry, 2003). *Eq. 4* can be rearranged (note that $m_{^{18}O} = m_{^{18}O1} + m_{^{18}O2}$) to read as (*Eq. 5*):

$$\frac{m_{^{18}O1}}{m_{^{18}O}} = \frac{\delta^{18}O-\delta^{18}O_2}{\delta^{18}O_1-\delta^{18}O_2} \quad \text{(Eq. 5)}$$

where $\frac{m_{^{18}O1}}{m_{^{18}O}}$ is the fraction contribution of tributary 1 to the mainstream.

The Dadu River is the most important tributary of the Minjiang River that in turn is a major tributary of the Yangtze River. The Dadu River has three sources: the east source Suomo River, the west source Duke River, and the main source Zumuzu River. At the confluence where the Sequ River merges into the Duke River, samples were taken and analyzed, and the following $\delta^{18}O$ values were obtained: -14.9‰, -14.0‰ and -14.2‰, respectively, from Sequ River, upstream of Duke River, and downstream of Duke River. Using *Eq. 5* it is calculated that $\frac{m_{^{18}O1}}{m_{^{18}O}}$ equals 0.28, i.e. the component from the Sequ River to the Duke River was 28% at the time of sampling.

Further upstream of the Sequ River, the Angkegou Stream in Rangtang is another tributary of the Duke River, but is much smaller than the Sequ River. The following $\delta^{18}O$ values were obtained around the point where the Angkegou Stream enters the Duke River: -14.9‰, -13.8‰ and -13.8‰, from the Angkegou Stream, upstream and downstream of the Duke River, respectively. Apparently, given the precision of our isotope analysis, it is not feasible to calculate the contribution of the Angkegou Stream to the Duke River using the oxygen isotope ratios with any confidence.

The Suomo River and the Duke River merge at Baiwan and then flow into the Dadu River. Near the joint, the $\delta^{18}O$ values were obtained as -13.1‰, -14.4‰ and -13.8‰, respectively, for the water samples from the Suomo River, Duke River and the Dadu River. The contribution of the Duke River to the Dadu River was calculated to be 55% and Suomo River to the Dadu River to be 45%.

The calculations based on mass balance are valid only when the tributary waters are fully mixed in the mainstream. Using the same method, the contribution of Ake River to

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Ma'erqu (a secondary tributary of the Yangtze River, upper reach of the Jiaomuzhu River) was calculated to be 75%; but it was noted during field work that the Ake River flow was too small and its contribution to the Ma'erqu River could not possibly be that high. This apparent error was likely due to the fact that the sample taken from downstream Ma'erqu River with $\delta^{18}O$ = -12.4 was most likely not representative of the well-mixed water body due to physical difficulty in sampling: a steep cliff prevented the sample from being taken near the center of the Ma'erqu River. Instead, the sample was taken near the cliff where the water was suspected to be predominantly from the Ake River ($\delta^{18}O$ = -12.2), and a thorough mixing with the Ma'erqu River water had not yet achieved. Note that these river mixing calculations are approximate, as the uncertainties in the δ-values (±0.2‰ and ±2.0‰ for $\delta^{18}O$ and $\delta^{2}H$, respectively) are not evaluated.

Conclusions

In general, the stable oxygen and hydrogen isotope ratios are correlated with the distance to the east coast of China, in agreement with a continentality model. That is, the stream waters were gradually depleted in heavy isotopes from east to west in the area studied. This phenomenon is attributed to the successive rainfalls from the same moisture cloud and the preservation of isotopic signature in the runoff from the precipitations. Local deviations from this general trend may be explained by the elevation effect and some anthropogenic factors.

The $\delta^{2}H$ and $\delta^{18}O$ data of the samples from the Yangtze River basin, which covers the majority of the study area, align well with the CMWL and particularly with the GMWL, suggesting that the stream waters in this area were primarily of meteoric origin (including ice and snow melting) with a regional weak evaporation effect prevailing. However, the data from a relatively small area belonging to the Yellow River basin are characterized by an appreciably flatter slope of the isotope water line, suggesting that the waters had undergone significant evaporation due to kinetic fractionations of isotopes in a more arid climate. These observations are also supported by the d-excess values.

Since the samples were taken during summer time, this study provides a snapshot of the region's river hydrology during the rainy season. As the stable isotope ratios can vary greatly both over time and space, for a more complete understanding of the local river hydrology more systematic sampling and analysis over time will be necessary. Precipitation samples should be collected and analyzed together with the stream water samples so that a direct correlation between the runoff waters and precipitations could be more firmly established. Nevertheless, the stream isotope data presented in this work should be valuable to those who are concerned with the TRH eco-system or, more broadly, the eco-environment of the Qinghai-Tibet Plateau.

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THE ROLE OF ENVIRONMENTAL FACTORS IN THE FORMATION OF ZOOPLANKTON IN TRIBUTARIES OF LAKE LADOGA (RUSSIA)

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Abstract. The role of environmental factors in the formation of zooplankton in tributaries of Lake Ladoga is still poorly investigated. The authors aimed at exploring composition and quantitative patterns of zooplankton development in the major tributaries of Ladoga and assessing the impact of environmental factors and features of river catchment basins on the parameters of zooplankton development. A total of 137 taxa ranking below genus were identified (56 species and subspecies of *Rotifera*, 57 of *Cladocera*, 24 of *Copepoda*). Species composition and patterns of quantitative development cannot be explained by any environmental factors or their combination. It is impossible to determine groups of rivers with similar patterns of zooplankton development basing on the composition and abundances of individual zooplankton species. River clustering based on hydrochemical and hydrological parameters and features of catchment basins provides an opportunity to distinguish groups of similar rivers. Total abundances of copepoda, cladocera, and rotifera in the rivers are closely associated with certain combinations of studied physiographic parameters. Physiographic factors, namely, the catchment basin area, water discharge, and lake percentage on the territory of the catchment basin, are the most important in the level of quantitative development of *Copepoda*, *Rotifera*, and *Cladocera* in tributaries of Lake Ladoga.
Keywords: *Copepoda, Cladocera, Rotifera, rivers, species composition, physiographic factors*

Introduction

Tributaries of Lake Ladoga greatly influence its water balance, hydrochemical water composition, biocenoses, and general ecological condition. River discharge counts for almost 85% of water balance input and more than 95% of chemical balance of the lake (Alekin, 1953). In river ecosystems, zooplankton community is an important structural and functional unit, for exampe, zooplankton organisms are a primary food source for larval and some adult fish (Thorp and Casper, 2003).

Zooplankton of lake tributaries have seldom been studied in comparison with zooplankton of lakes and reservoirs (Thorp and Mantovani, 2005; Turschak et al., 2011). Development of zooplankton in rivers is controlled by a still poorly understood mixture of abiotic and biotic factors varying seasonally and correlated to chemical and physical features of the rivers and physiographic conditions of their drainage basins (Thorp and Casper, 2002; Zhao et al., 2017).

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Planktonic crustaceans and rotifers play a big part in the process of transformation and circulation of organic matter and so participate in river self-purification (Krylov, 2002a; Ejsmont-Karabin et al., 2004).

Contrary to thoroughly studied zooplankton of Lake Ladoga (Andronikova, 1996; Kurashov et al., 1996; Telesh, 1996; Korovchinsky, 2000), the zooplankton of its tributaries has not been properly studied.

Literature contains limited data on zooplankton communities of some rivers of the Northern Ladoga area (Ryzhkov, 1999; Kulikova, 2012; Ryabinkina et al., 2012) and main tributaries (Makartseva, 2004; Kulikova, 2012; Aleshina et al., 2014). These data have been collected with the help of different methods at various times, therefore, the results cannot be properly compared, making it impossible to assess the influence of environmental factors on the communities of crustaceans and rotifers. It is vital to determine the patterns of this influence to provide a better understanding of the processes taking place in river ecosystems and opportunity to model and predict the zooplankton reaction to environmental changes, including those connected with current climate changes (Vadadi-Fülöp et al., 2009).

Previous studies of the Lake Ladoga tributaries have shown that the development of phytoplankton in rivers depends on the supply with nutrients, first of all, phosphorus, and on factors such as the productivity in the source of the river, character and rate of river flow, presence or absence of lakes or widened stretches with retarded water exchange in the course of the river (Trifonova and Pavlova, 2004). The role of environmental factors in the formation of zooplankton in tributaries of Lake Ladoga is still poorly investigated.

In this study, we aimed to explore qualitative composition and quantitative patterns of development of zooplankton in the major tributaries of Lake Ladoga, and to assess the impact of environmental factors and features of river catchment basins on the parameters of zooplankton development.

In the face of constantly increasing anthropogenic load on the Lake Ladoga catchment basin (Kondratyev and Trumbull, 2012), it is important to reveal interactions between environmental factors and the state of biota. In this connection, the results of the current study can be used to develop the strategy for management and preservation of water resources of the Ladoga area rivers.

Materials and Methods

Study area

Three major tributaries can be determined within the catchment basin of Lake Ladoga: the Svir, the Volkhov, and the Vuoksa system of lakes and rivers, entering Lake Ladoga in two branches – the northern one, Vuoksa River, and the southern one, Burnaya River. Second-order tributaries are the Pasha, the Oyat, the Syas, and the Olonka rivers. Although the Pasha and the Oyat are tributaries of the Svir, they are considered to be independent lake tributaries, as they enter the Svir near its mouth. There are 20 major water courses entering Ladoga (*Table 1*), including the Burnaya and the Vuoksa, which actually belong to the same Vuoksa system of lakes and rivers. For our study in 2011–2014 we chose the stations at the lower course of these rivers and at the Neva River head (*Fig. 1*).

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Material and data analysis

Our study included 9 periods: May, August, and October 2011, July and October 2012, June and September 2013, May and July 2014. Hydrochemical data were obtained with the help of YSI 6600D multiparameter automatic sonde (YSI Incorporated, USA) directly during the zooplankton sampling within the surface layers or assessed with routine methods at the laboratory (Semenov (ed.), 1977). The following parameters were measured and used for the statistical analyses (abbreviations are given for the variables that were later included in the regression equations): content of aluminium (Al, µg/l), copper (Cu, µg/l), iron (Fe, µg/l), lead (Pb, µg/l), zinc (Zn, µg/l), sodium and potassium (Na+K, µg/l), manganese (Mn, µg/l), ions of calcium (Ca^{2+}, µg/l) and magnesium (Mg^{2+}, µg/l), ammonium (NH_4^+, mg/l), chlorides (Cl^-, mg/l), nitrates (NO_3^-, mg/l), sulphates (SO_4^{2-}, mg/l), carbonates (HCO_3^-, mg/l), total nitrogen (N_{tot}, mg/l) and phosphorus (P_{tot}, mg/l), inorganic phosphorus (P_{min}, mg/l), total carbon (TOC, mgC/l), biological oxygen demand in 5 days (BOD_5, mgO_2/l), chemical oxygen demand (COD, mgO_2/l), hardness of water, content of hydrocarbons (Carb, mg/l), content of oxygen (percent saturation and mg/l), content of total suspended matter, water colour (W_{col}, degrees), turbidity (NTU), acidity (pH), specific conductance (Cond, mS/cm), total dissolved solids (TDS, g/l), temperature, content of chlorophyll (Chl, µg/l) and cyanobacteria abundance (BGA, cells/ml).

***Table 1.** Characteristics of catchments and the long-term average discharges of the studied rivers (Tarakanova, 1965).*

№	River	The catchment area, km²	Lake percentage on the territory, %	Intensity of waterlogging, %	Length, km	Water discharges, m^3/s
1	Neva	281000	0	0	74	2500.0
2	Volkhov	13000	0	8.7	224	569.0
3	Svir	9820	1.0	14.0	224	653.0
4	Syas	7330	1.0	16.0	260	65.0
5	Burnaya*	7130	10.0	3.6	156	642.0
6	Vuoksa*	7130	10.0	3.6	156	642.0
7	Pasha	6650	1.0	18.0	242	78.0
8	Oyat	5220	3.0	11.0	266	59.0
9	Yanis	3900	14.0	5.0	126	41.7
10	Olonka	2620	3.0	10.0	87	35.0
11	Tulema	1720	5.0	16.0	55	21.8
12	Tohma	1602	6.0	9.6	74	9.2
13	Kokkalan	1370	14.0	4.0	60	10.7
14	Vidlitsa	1320	9.0	9.0	67	18.5
15	Uksun	1080	6.0	9.0	121	15.0
16	Tuloksa	900	0	11.0	77	8.6
17	Lava	529	3.3	0	31	4.2
18	Morje	478	0	0	43	4.0
19	Avloga	385	2.0	0	54	1.0
20	Nasiya	332	0.8	0	42	2.1

* – data for a single river-lake system Vuoksa.

The following parameters of catchment basins of Lake Ladoga major tributaries were used: river length (L, km), areas of wetlands (Sh_{wetl}, %), lake percentage on the territory of the catchment basin (Sh_{lakes}, %), catchment area (S_{cat}, km^2), river flow rate (W_{fl}, m^3/s) (*Table 1*).

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Zooplankton samples were collected from the shore at open areas lacking macrophytes by pouring 100 L of water through the Apstein cone-shaped net (mesh size is 75 μm) and fixed in 40% formalin (diluted to 4.0%). Species composition and qualitative development patterns of rotifers of the tributaries zooplankton were assessed after processing the sediment samples. The material was processed according to the routine protocols described by Abakumov (1992). Biomass of the organisms (raw formalin weight) was calculated using the formulas for the dependence between body length and weight of planktonic crustaceans (Ruttner-Kolisko, 1977; Balushkina and Vinberg, 1979). Species composition was determined with the help of identification manuals (Rylov, 1963; Kutikova, 1970; Alekseev and Tsalolikhin (eds.), 2010).

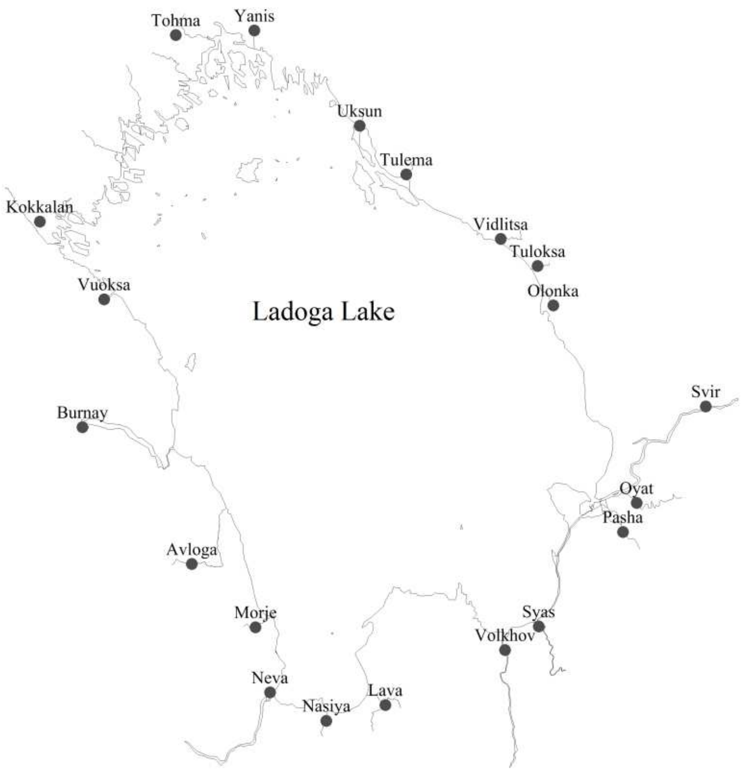

Figure 1. *Scheme of sampling stations on the tributaries of Lake Ladoga and Neva River*

The following parameters were used to characterize the zooplankton community: number of species (n), abundance (N, ind./m^3), biomass (B, mg/m^3), the Shannon diversity index (Shannon, 1948) estimated for both abundance (H_N, bit/ind.) and biomass (B_N, bit/mg). To separate groups of rivers, similar in zooplankton, the numbers of individual species of Copepoda, Cladocera, and Rotifera, and members of Copepoda, Cladocera, and Rotifera groups that have not been identified to species (Copepodits of Calanoida, Copepodits of Cyclopoida, Nauplii of Calanoida, Nauplii of Cyclopoida, Cyclopoida spp.) (86 variables in total), as well as total numbers of Copepoda, Cladocera, and Rotifera were used.

Statistical analysis of the variables was performed for each time period. Cluster analysis, correlation analysis and regression analysis (stepwise multiple regression) included in Statistica 6.0 software package (StatSoft Inc.) were used to reveal

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interrelationships between the studied parameters. The authors used the modification of cluster analysis, where the variables correlation measure was Euclidean distance or Pearson correlation coefficient; individual rivers were included into clusters according to the complete linkage method or Ward method (Pesenko, 1982).

Results

Chemical composition

Chemical composition of water in tributaries of Lake Ladoga is typical for the area under study, its common features result from similar climate conditions, and the differences are caused by peculiarities of geomorphologic characteristics, the composition of the rocks (*Appendix A*).

The waters of tributaries of Lake Ladoga fall into the hydrocarbonate class of calcium group. Calcium ions prevail in the cationic composition of water of all rivers – 1.6 to 52.63 µg/L, the content of magnesium ions varies from 0.7 to 16.2 µg/L, the content of sodium and potassium ions – from 0.08 to 19.95 µg/L. The content of sulphates varies from 1.82 mg/L (the Uksun River) to 17.00 mg/L (the Kokkolan River), the content of chlorides – from 1.03 mg/L (the Uksun River) to 19.14 mg/L (the Volkhov River), the content of carbonates – from 0.73 mg/L (the Uksun River) to 185.93 mg/L (the Lava River). During the period of studies, the water temperature was above 3.5ºC and below 22.5ºC.

Community composition and quantitative patterns of zooplankton development

In the investigated rivers, a total of 137 zooplankton taxa ranking below genus were identified in the lower course of the Lake Ladoga tributaries and the Neva River head. Rotifera group accounted for 56 species and subspecies (40.9%), Cladocera – 57 (41.6%), Copepoda – 24 (17.5%), including 6 members of Calanoida (4.4%), 18 – Cyclopoida (13.1%). The greatest number of species was found in large rivers: the Vuoksa (57), the Burnaya (51), the Volkhov (49), the Svir (45), the Neva (45), the lowest number – in the Kokkolan River (26) (*Appendix B*).

Species composition considerably varied between different rivers. *A. harpae*, *B. (E.) coregoni*, *C. sphaericus* were the only crustaceans found in all rivers. Almost every tributary was inhabited by following zooplankters: *A. priodonta* (except for the Naziya River), *E. lyra* (except for the Pasha River), *B. (B.) longirostris* (except for the Vidlitsa River), *M. leuckarti* (except for the Tokhma River and the Vidlitsa River). Such members of lower crustaceans as *B. (E.) coregoni* (frequency of occurrence, F = 11.5%), *C. sphaericus* (F = 11.5%), *B. (B.) longirostris* (F = 9.6%), *A. harpae* (F = 9.3%), *P. pediculus* (F = 9.3%), *D. cristata* Sars (F = 7.6%), and such rotifers as *Synchaeta spp.* (F = 12.4%), *A. priodonta* (F = 10.4%), *C. unicornis* (F = 7.6%), *K. longispina* (F = 7.1%), *Bdelloida spp.* (F = 5.4%), *B. hudsoni* (F = 4.3%), *E. incisa* (F = 3.8%), *K. quadrata* (F = 3.2%), *A. herricki* (F = 2.7%) were the most abundant in the zooplankton samples.

As a rule, several species simultaneously prevailed in the rivers. Among Cladocera *B. (E.) coregoni* was dominant more often than any other zooplankters (*Table 3*). The members of the lake complex – *A. priodonta*, *K. longispina*, *C. unicornis, D. (D.) cristata, E. gracilis*, *T. oithonoides*, as well as the crustaceans of the littoral macrophyte complex – *A. harpae*, *A. quadrangularis*, *C. sphaericus* prevailed in most tributaries.

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Plankton community was formed by 53 cosmopolitan species (43.8%), 32 holarctic species (25.4%), 31 palaearctic species (25.6%), 5 boreal species (4.1%).

The highest numbers of zooplankton within the Lake Ladoga tributaries were observed in the Avloga River in May 2011 (9580 ind. /m^3), in the Tuloksa River and the Burnaya River in June 2014 (9190 ind./m^3 each). The highest levels of biomass development were observed in the zooplankton communities of the Vuoksa River in May 2015 (266.2 mg/m^3), in the Tuloksa River in June 2014 (256.5 mg/m^3). The lowest levels of population and biomass (30 ind./m^3 and 0.17 mg/m^3 respectively) were observed in small rivers (the Tokhma River, the Uksun River). The mean levels of population in the tributaries during the study period of 2011–2014 varied from high – 2870 ± 670 ind./m^3 in the Vuoksa River – to low – 210 ± 50 ind./m^3 in the Lava River, the levels of biomass – from 191.77 ± 125.22 mg/m^3 in the Vuoksa River to 1.84 ± 0.55 mg/m^3 in the Kokkolan River (*Table 2*).

Table 2. *Mean abundance (N_{tot}, ind/m^3); biomass (B_{tot}, mg/m^3) and the values of the Shannon index, calculated on the number (H_N, bit/ind.) and biomass (H_B, bit/mg) of zooplankton species in the tributaries of Lake Ladoga and the Neva river in 2011–2014.*

№	River	N_{tot}, ind/m^3	B_{tot}, mg/m^3	H_N, bit/ind.	H_B, bit/mg
1	Neva	6910±3320	123.87±53.97	2.24±0.10	1.88±0.14
		1.44	1.31	0.13	0.22
2	Volkhov	410±130	18.19±8.28	1.84±0.16	1.52±0.18
		0.91	1.37	0.25	0.37
3	Svir	1810±880	46.72±22.11	1.84±0.25	1.63±0.20
		1.45	1.420	0.415	0.36
4	Syas	700±230	25.82±20.23	1.66±0.17	1.48±0.17
		0.97	2.35	0.31	0.34
5	Burnaya	2680±1000	62.58±27.93	2.30±0.16	2.17±0.13
		1.12	1.34	0.21	0.18
6	Vuoksa	2870±670	191.77±125.22	2.40±0.15	2.14±0.16
		0.70	1.96	0.19	0.22
7	Pasha	420±110	13.11±10.12	1.63±0.27	1.47±0.23
		0.76	2.32	0.49	0.48
8	Oyat	660±180	9.17±2.99	1.80±0.20	1.65±0.16
		0.81	0.98	0.33	0.29
9	Yanis	410±80	8.38±2.34	1.88±0.27	1.63±0.28
		0.61	0.84	0.44	0.51
10	Olonka	1070±410	17.46±4.67	2.06±0.14	1.88±0.14
		1.17	0.80	0.20	0.22
11	Tulema	860±460	9.95±4.27	1.89±0.21	1.48±0.19
		1.60	1.29	0.33	0.38
12	Tohma	290±90	5.28±1.68	1.67±0.25	1.32±0.20
		0.90	0.96	0.45	0.46

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№	River	N_{tot}, ind/m³	B_{tot}, mg/m³	H_N, bit/ind.	H_B, bit/mg
13	Kokkalan	180±40 0.66	1.84±0.55 0.90	1.54±0.15 0.29	1.34±0.21 0.48
14	Vidlitsa	270±80 0.87	4.48±1.83 1.23	1.45±0.17 0.36	1.10±0.15 0.40
15	Uksun	540±290 1.61	7.17±3.43 1.43	1.89±0.21 0.34	1.42±0.28 0.58
16	Tuloksa	1990±1210 1.72	68.97±35.77 1.56	1.67±0.20 0.36	1.41±0.22 0.46
17	Lava	210±50 0.65	3.47±1.87 1.42	1.86±0.12 0.17	1.58±0.24 0.40
18	Morje	500±150 0.87	7.52±2.23 0.89	1.94±0.15 0.23	1.75±0.19 0.33
19	Avloga	1450±1030 2.13	8.08±3.54 1.31	1.52±0.18 0.35	1.25±0.13 0.30
20	Nasiya	350±100 0.89	5.99±2.07 1.04	1.74±0.15 0.26	1.48±0.16 0.32

Note: above the line – the arithmetic mean ± standard error of the mean, below the line – the coefficient of variation of the characteristics.

The highest numbers of population and biomass at the station of the Neva River head were observed in August 2011 (29360 ind./m^3 and 468.3 mg/m^3 respectively), whereas the lowest ones – in May 2015 (480 ind./m^3 and 4.65 mg/m^3 respectively). Mean value of zooplankton abundance was 6910 ± 3320 ind./m^3, mean biomass – 123.87 ± 53.97 mg/m^3 (*Table 2*).

The values of the Shannon index for zooplankton abundance (H_N) varied from 0.29 bit/ind. (the Pasha River) to 3.07 bit/ind. (the Vuoksa River) and from 0.18 bit/mg (the Yanis River) to 2.89 bit/mg (the Vuoksa River) for zooplankton biomass (B_N).

Mean values of Rotifera abundance were higher than those of the crustaceans in the Tuloksa and the Morje rivers, mean biomass – in the Burnaya, the Tuloksa, the Morje, the Kokkolan rivers. The Copepoda crustaceans prevailed in both number and biomass in the zooplankton communities of the Neva head and the Volkhov mouth. Mean values of number and biomass of Cladocera were higher than total values of other zooplankton groups in the rest of the rivers (*Figs. 2, 3*).

Statistical analysis

Cluster analysis of the variables was performed for 9 time periods: May, August, and October 2011, July and October 2012, June and September 2013, May and July 2014.

When clustering rivers according to the species composition and species abundance, no similar clusters could be formed both in different seasons of the same year and in different years (*Figs. 4–7*).

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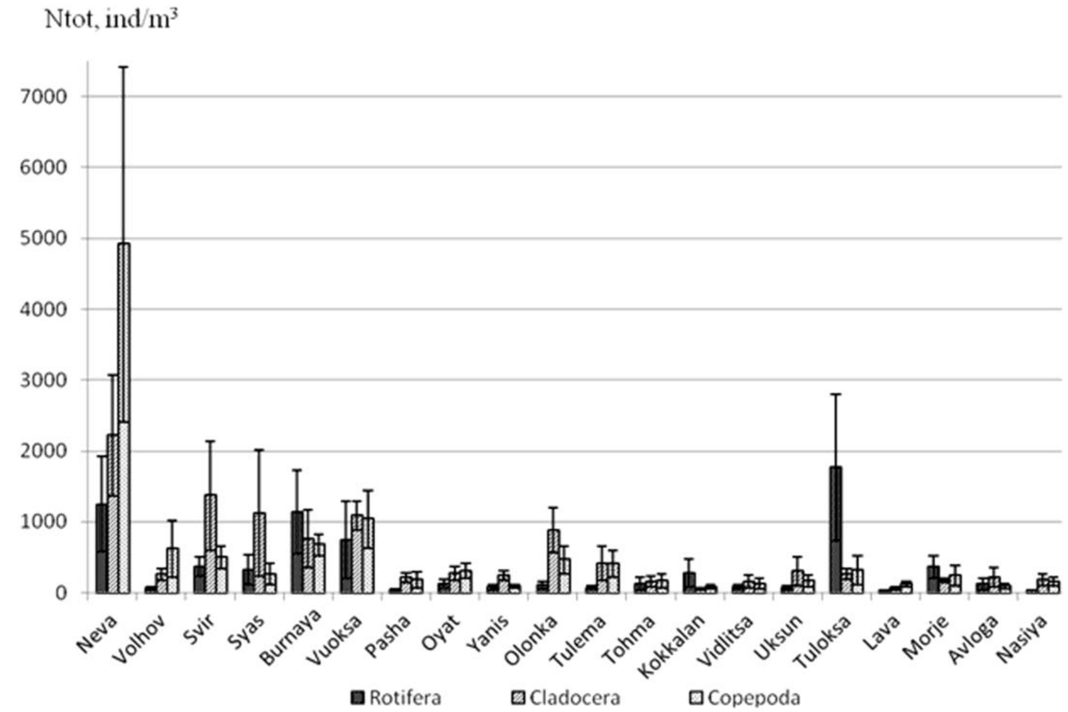

Figure 2. *The average values of the number of major zooplankton groups in 2011–2014*

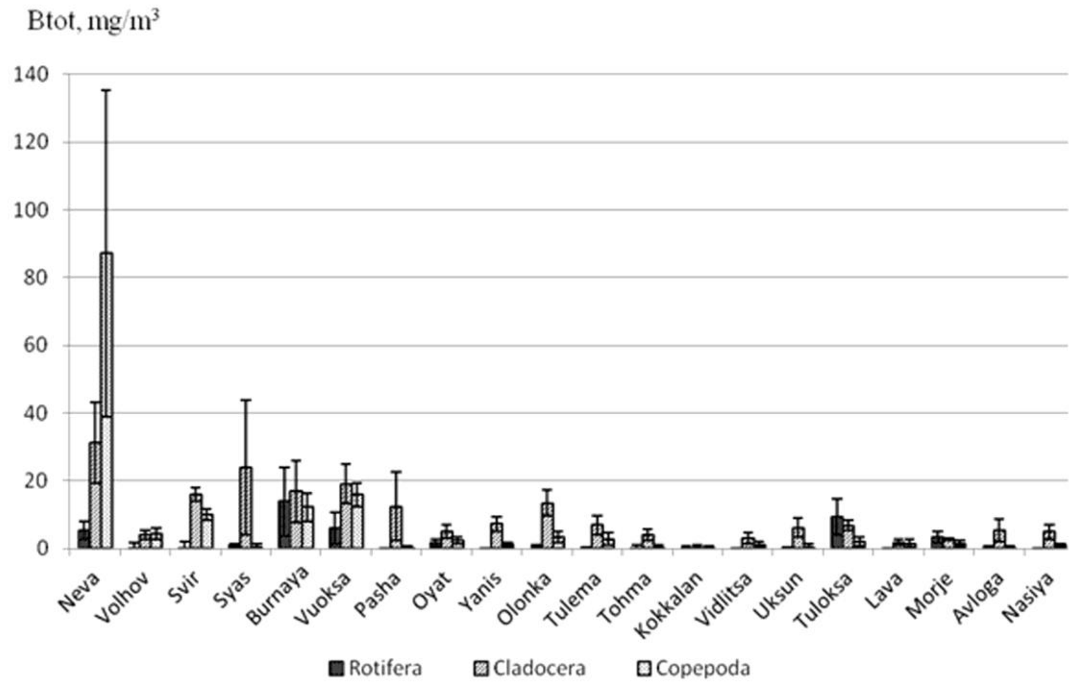

Figure 3. *The average values of the biomass of major zooplankton groups in 2011–2014*

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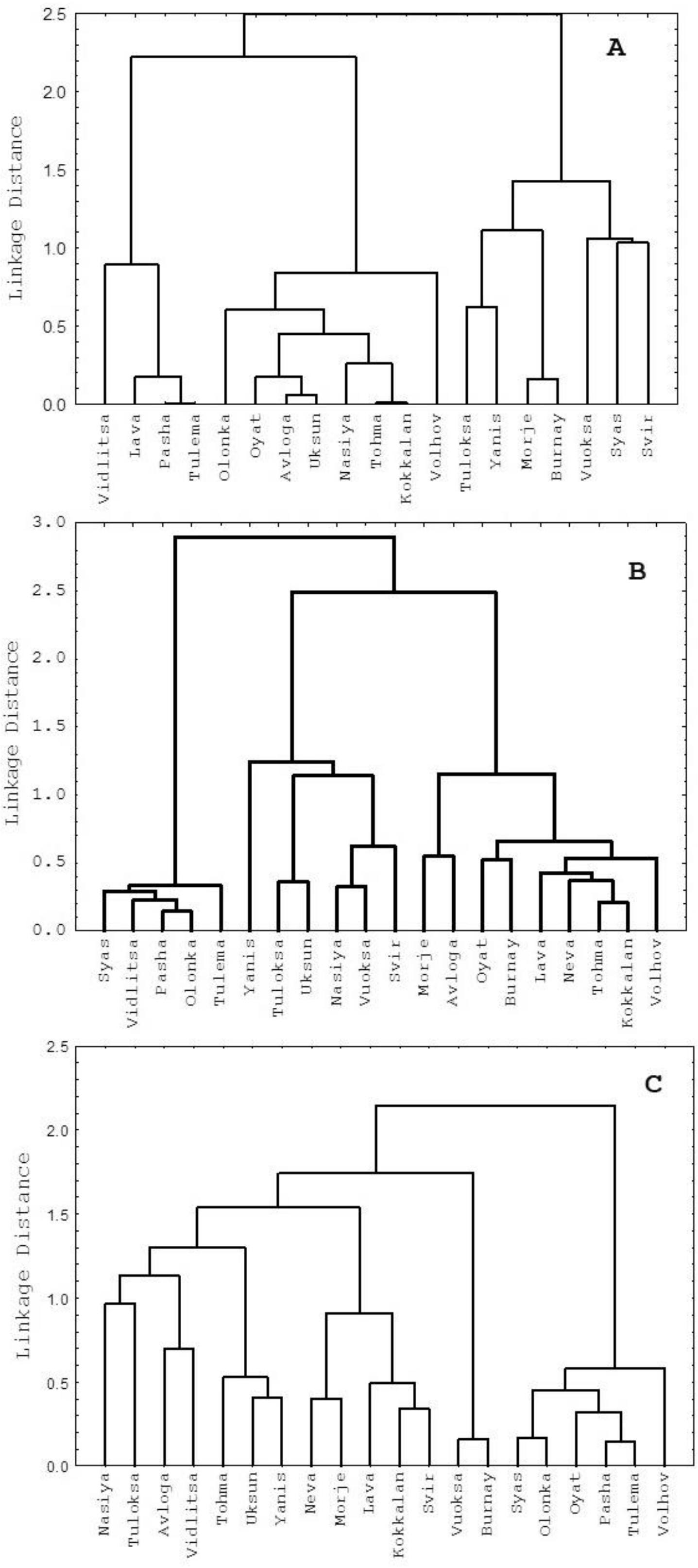

Figure 4. *Diagram of similarities of tributaries of Lake Ladoga in 2011, based on the analysis of the composition and abundance of zooplankton species (joining by the Ward's method, distance measure – Pearson correlation coefficient). A – May, B – August, C – October.*

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River groups of the Vidlitsa-Pasha-Tulema and the Tokhma-Kokkolan-Volkhov showed similar trends of clustering in spring and summer 2011; the Lava-Kokkolan and the Syas-Olonka – in summer and autumn; the Pasha-Tulema – in spring and autumn (*Fig. 4*).

In 2012, the rivers did not form similar clusters for different time periods, when the analysis was based on the numbers of individual zooplankton species (*Fig. 5*).

In 2013, similar trends of clustering were observed in case of the Kokkolan-Volkhov rivers in summer and autumn (*Fig. 6*).

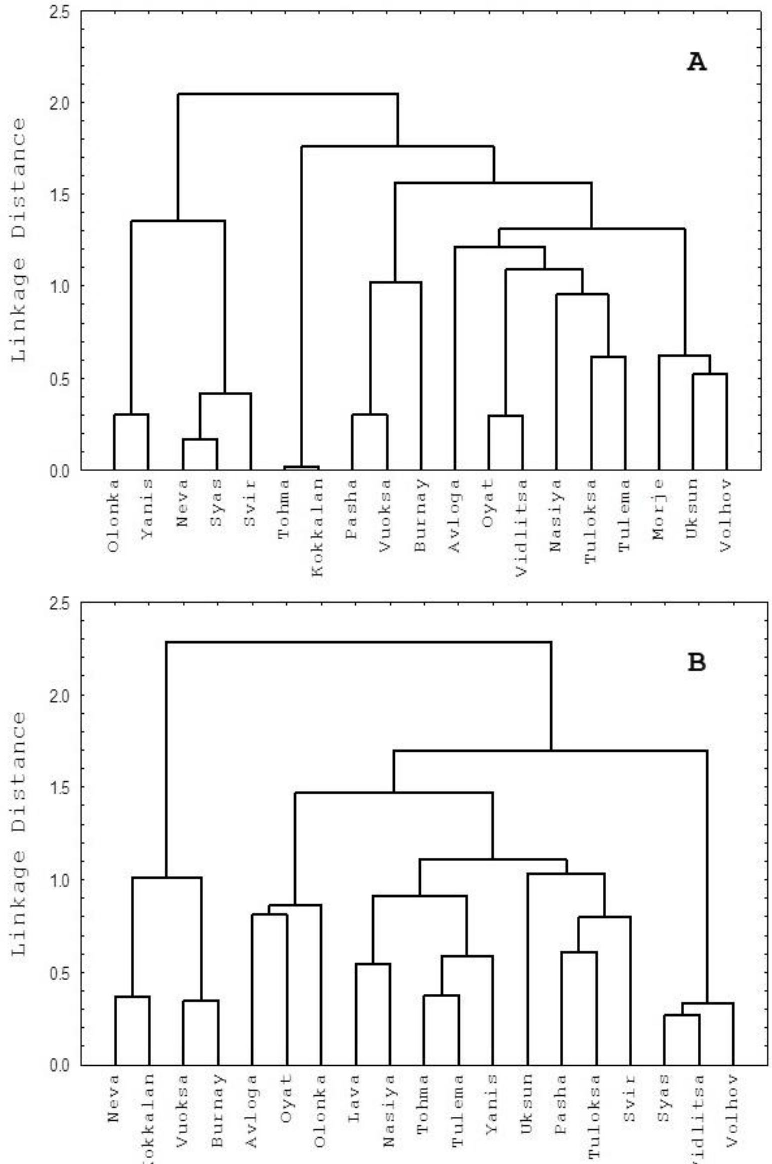

Figure 5. *Diagram of similarities of tributaries of Lake Ladoga in 2012, based on the analysis of the composition and abundance of zooplankton species (joining by the Ward's method, distance measure – Pearson correlation coefficient). A – July, B – October.*

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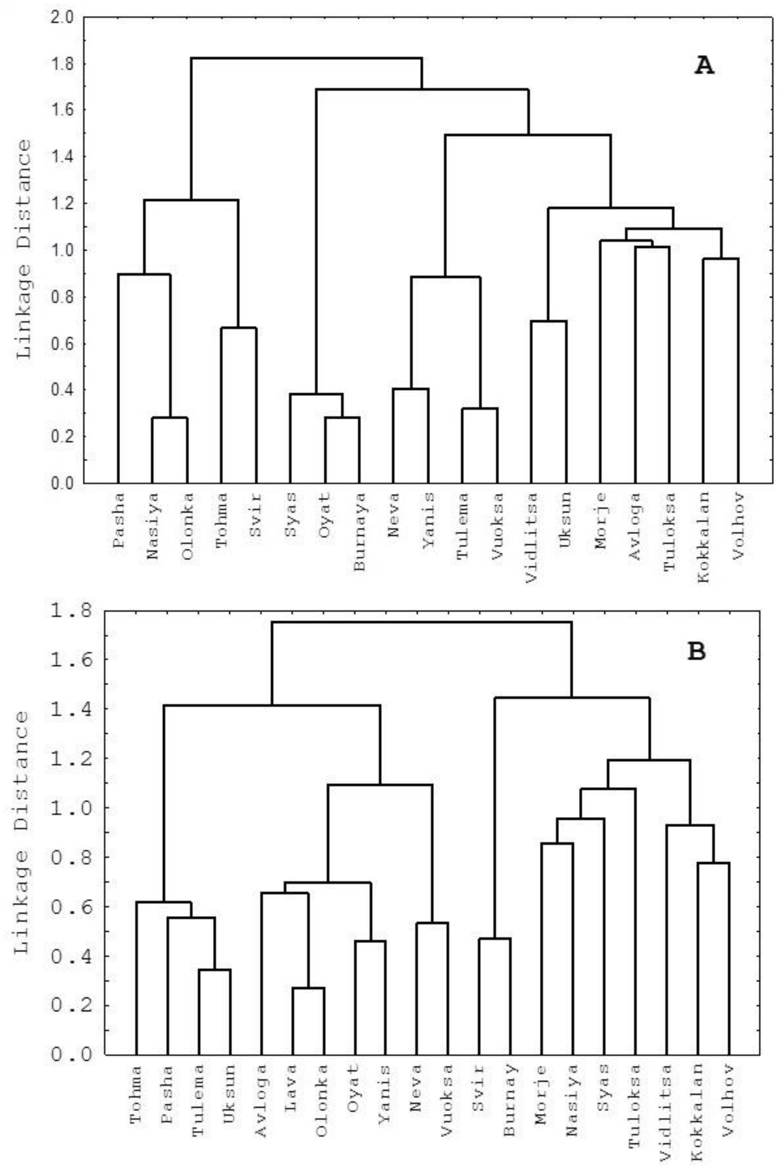

Figure 6. *Diagram of similarities of tributaries of Lake Ladoga in 2013, based on the analysis of the composition and abundance of zooplankton species (joining by the Ward's method, distance measure – Pearson correlation coefficient). A – June, B – September.*

In 2014, similar trends of clustering were only observed in case of the Lava-Pasha rivers and the Oyat-Naziya-Volkhov rivers in spring and summer (*Fig. 7*).

The examples of river clustering based on the hydrochemical, hydrological parameters and the catchment basin features (4 periods of 9) are shown in *Fig. 8*.

In general, the clusters of rivers similar in zooplankton communities did not coincide with the clusters of rivers that considered all the hydrochemical, hydrological parameters and the catchment basin features, in any year of studies (*Fig. 8*).

Generally, the clustering based on the hydrochemical, hydrological parameters and the catchment basin features was consistent in different years and seasons. Similar rivers were grouped according to their physiographic position (*Fig. 8*).

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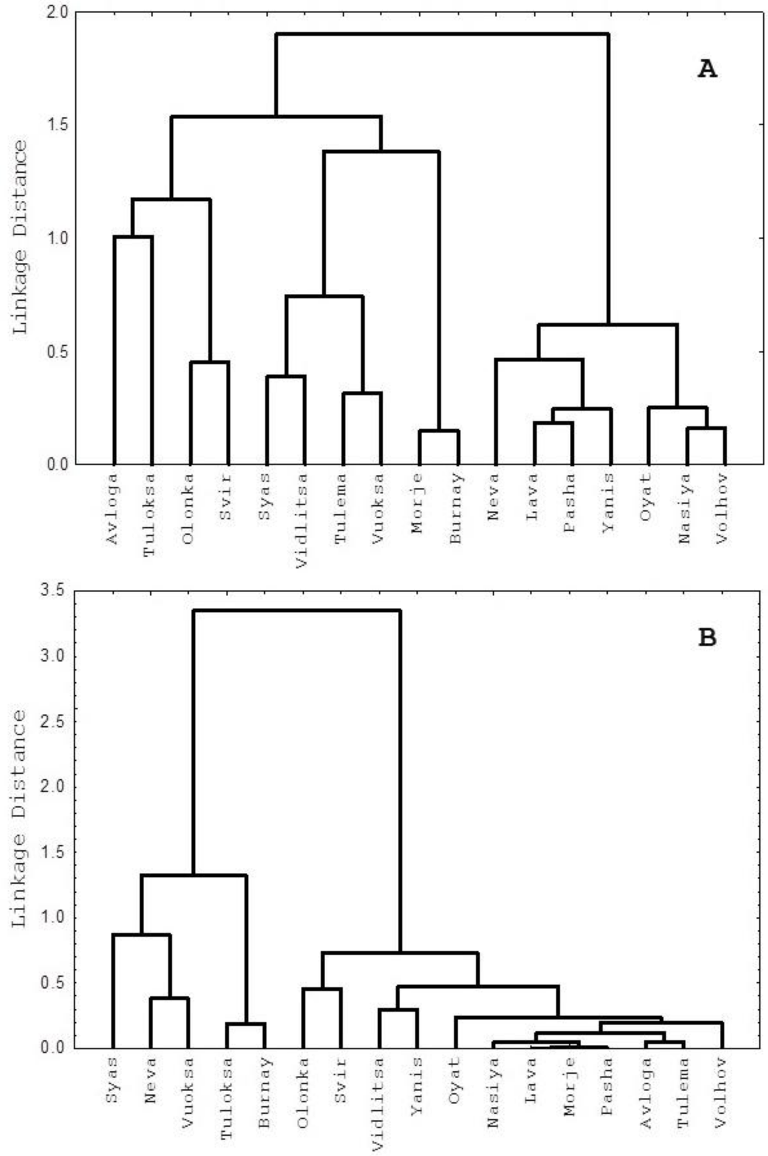

Figure 7. *Diagram of similarities of tributaries of Lake Ladoga in 2014, based on the analysis of the composition and abundance of zooplankton species (joining by the Ward's method, distance measure – Pearson correlation coefficient). A – May, B – July.*

The results of regression and correlation analysis did not show significant ($p<0.05$) relationship between the parameters of the development of individual zooplankton species and the assessed parameters of water of the Lake Ladoga tributaries.

Unlike the regression analysis that failed to show connection between the development of zooplankton species and the environmental factors, and to provide significant ($p<0.05$) equations of regression, the analysis of total numbers of Copepoda, Cladocera, and Rotifera resulted in highly significant correlations. 8 of 9 possible significant regression equations describing the group size as a function of a certain set of variables were obtained for Copepoda, 7 – for Rotifera, 6 – for Cladocera (*Table 3*).

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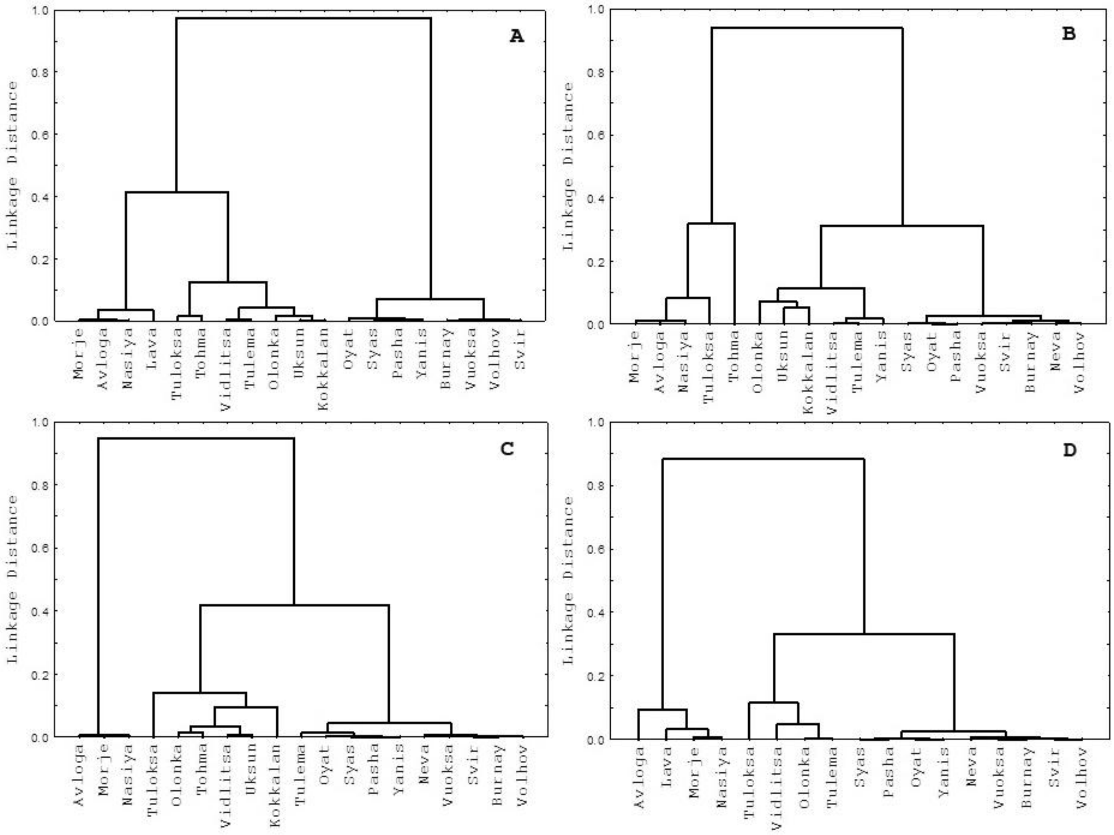

Figure 8. *Diagrams of similarities of tributaries of Lake Ladoga in different years, based on the analysis of the hydrochemical, hydrological parameters and characteristics of the catchment. (A: May 2011; B: July 2012; C: June 2013; D: May 2014) (joining by the Complete Linkage, distance measure – Pearson coefficient).*

The coefficient of determination (adjusted R^2) for the resulting equations varied from 0.4975 to 1.000 (*Table 3*). The range of the regression significance (p) was 0.0095–0.0000. The examples of the obtained regressions for each year and group are shown in *Figs. 9–12*.

Table 3. *The number of significant regressions ($p < 0.05$) (max = 9), the range of adjusted R^2 and the range of significance of obtained regression (p) for Copepoda, Cladocera and Rotifera in the tributaries of Lake Ladoga.*

Group	**The number of significant regressions ($p < 0.05$)**	**The range of adjusted R^2**	**The range of significance of regressions (p)**
Copepoda	8	0.5570–1.0000	0.0032–0.0000
Rotifera	7	0.6322–1.0000	0.0095–0.0000
Cladocera	6	0.4975–0.9999	0.0095–0.0000

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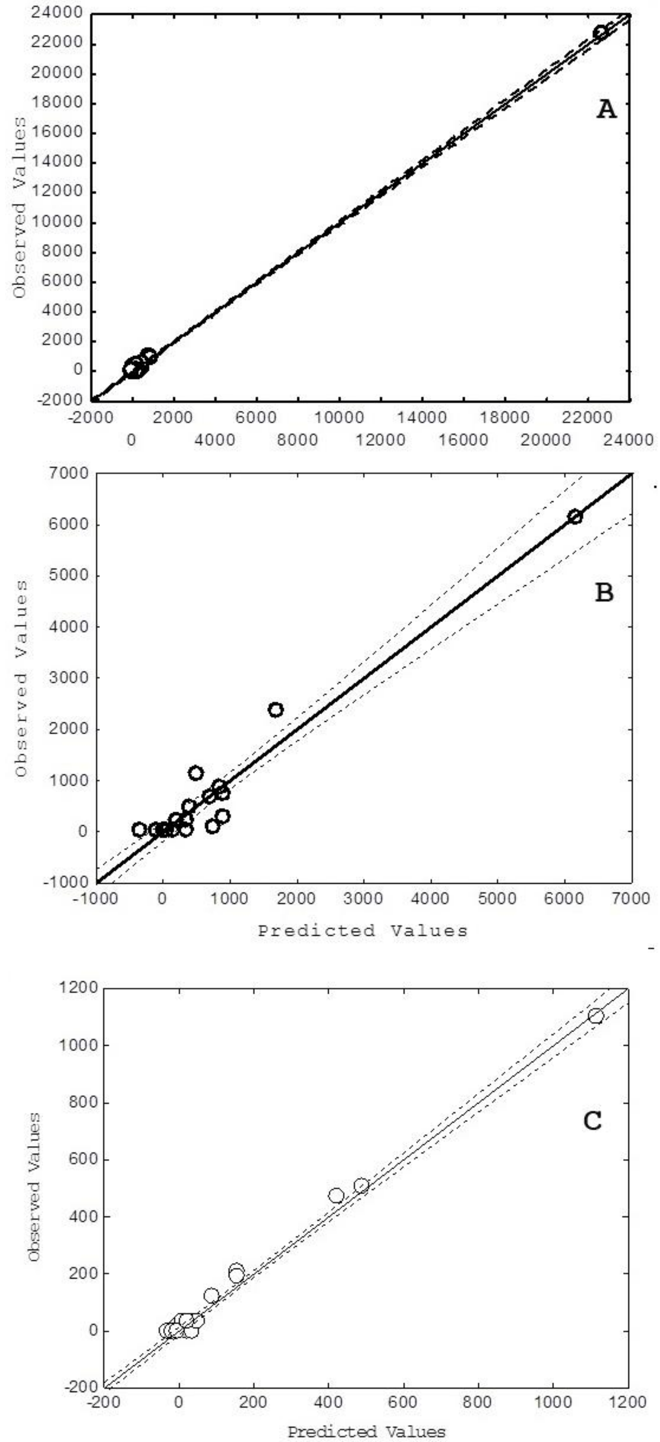

Figure 9. *Regression models relating the number of the groups of zooplankton with environmental factors for 2011:*

A: Copepoda = $0.08S_{cat}$-2761Mg+1132pH+0.19Fe+26.07Wet+$982SO_4$-7940 (August. $R^2 = 0.9984$); B: Cladocera = 0.02Scat-3877Mg+2580(Na+K)-0.76Al-164Cu+5512Cl+$916SO_4$+$80.27Sh_{wetl}$+3.78Wcol+110 (August. $R^2 = 0.8909$); C: Rotifera = $5186SO_4$-$1.353W_{fl}$+2424Cl-0.698Al+6.439L+$2.460W_{col}$+2498Ca-0.098Fe + $0.013S_{cat}$+4711(Na+K)-$3.485P_{tot}$-38.105TDS-$24.373Sh_{lakes}$+14.15COD-27.602Cu-179Pb-768 (August. $R^2 = 0.9464$).

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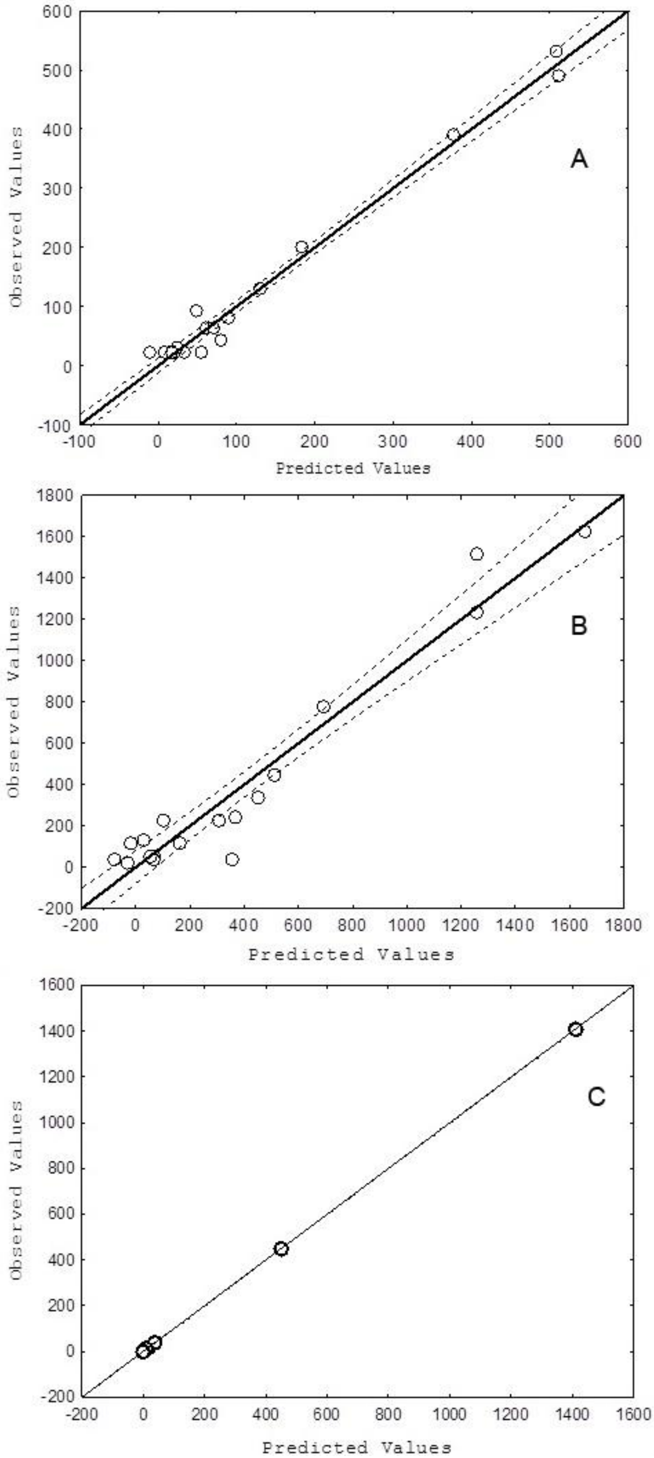

Figure 10. *Regression models relating the number of the groups of zooplankton with environmental factors for 2012:*

A: Copepoda = $0.442W_{col}$-56Ca-$0.006S_{cat}$+$0.797W_{fl}$+$11.86HCO_3$-$7.95Sh_{wetl}$-24.94Pb-6.290Cu+$14.99Sh_{lakes}$+0.06Fe-0.396Mn-18 (July. R^2 = 0.9614); B: Cladocera = +$1.084W_{fl}$ +$1.968P_{tot}$+$63.12Sh_{lakes}$-1.861BGA-42.84Cu+2.126Mn+$1.32W_{col}$+99.22Pb-697 (July. R^2 = 0.8939); C: Rotifera = 159.1Chl-6.5BGA+15.9ORP-0.4Fe+2318pH-$47.9Sh_{wetl}$-$21.7HCO_3$+$0.009S_{cat}$+0.4L+113.9Cu+$180.2SO_4$-204.9Pb+$1502NH_4$-47.8(Na+K)-$11.3Sh_{lakes}$-3758TDS+34.7Mg-1.4Cl-18631 (July. R^2 = 1.000).

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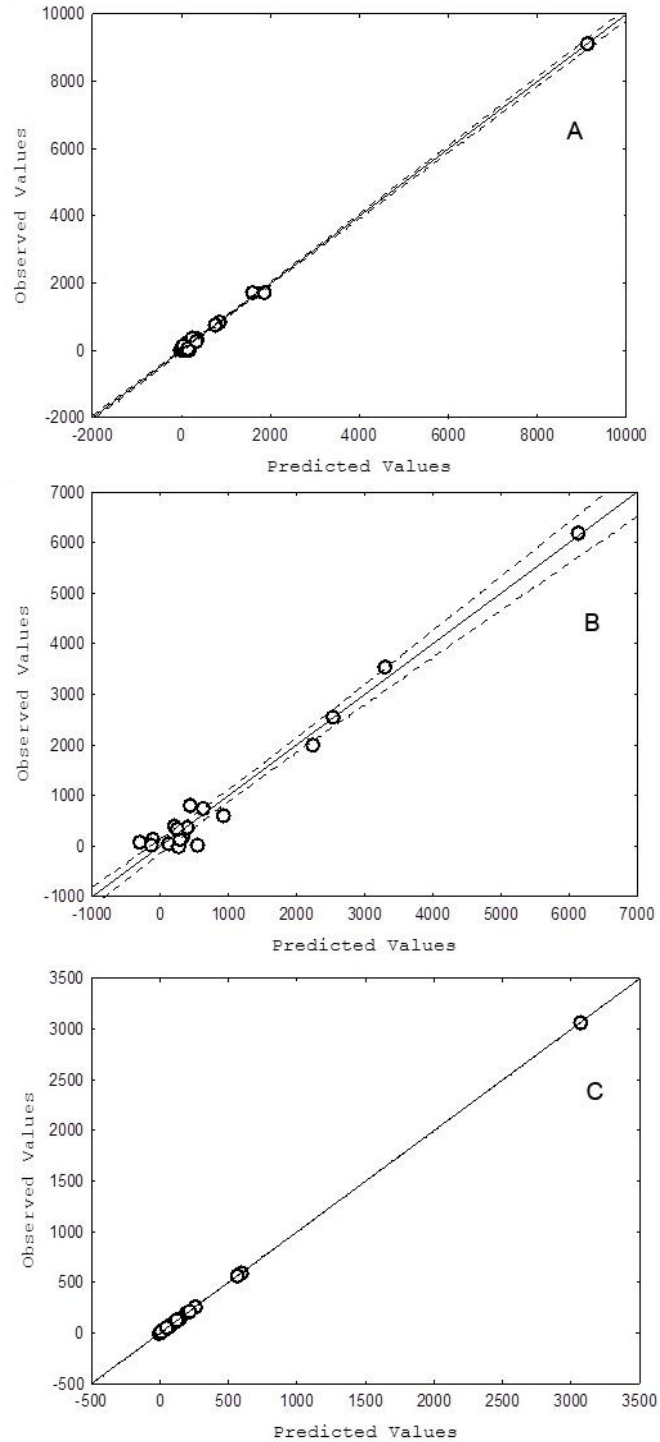

Figure 11. *Regression models relating the number of the groups of zooplankton with environmental factors for 2013:*

A: Copepoda = $0.014S_{cat}$-2114pH-$30.15SO_4$-1679Zn-11.11L+$143.54Sh_{wetl}$+$2.78W_{fl}$ +128.91(Na+K)+3.44Al-$2327N_{tot}$+29.04COD+$8.32P_{tot}$+16927 (September. R^2 = 0.9959); B: Cladocera = $5.16W_{fl}$ -$0.05S_{cat}$+84.66Cl+1.29Fe-441Chl-$289Sh_{lakes}$-193(Na+K)-$12.07P_{tot}$-305BGA+1.34Al+5283(June. R^2 = 0.9487); C: Rotifera = 14718Pb-33.84TOC+494Zn+1.77L+$51Sh_{wetl}$+61Cl+97Chl-202Ca+288Mg-0.34Fe+$39.88SO_4$+$25.57Sh_{lakes}$+180pH+0.14Al+93Cu+$0.004S_{cat}$+$3.52P_{tot}$+233TDS-10168 (September. R^2 = 0.9999).

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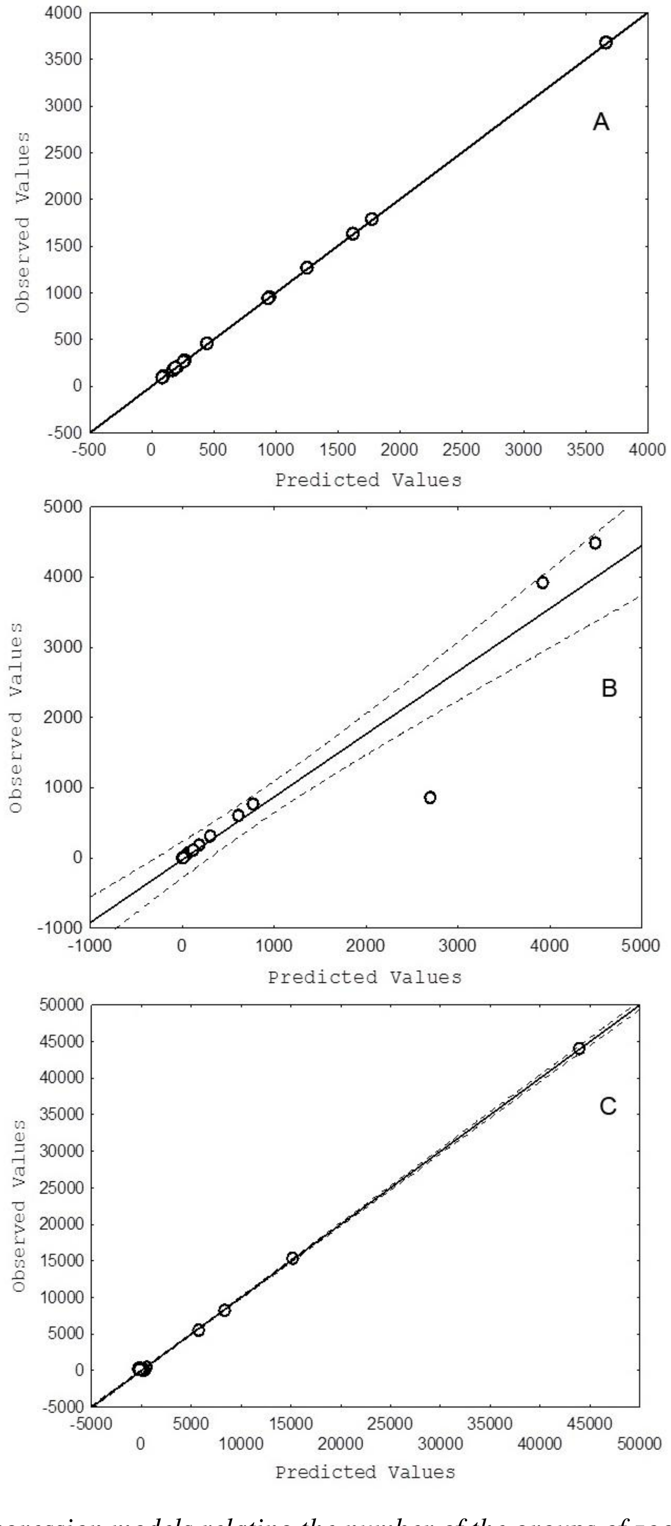

***Figure 12.** Regression models relating the number of the groups of zooplankton with environmental factors for 2014:*

A: Copepoda = $4382NH_4+24.28NTU-336Mg+1327pH+914Pb-0.03S_{cat}+3.27W_{fl}-4.39L+4.04W_{col}+6.05Mn-814N_{tot}-103NO_3+44.36(Na+K)-29.51BOD_5-0.08P_{tot}-9283$ (May. $R^2 = 1.0000$); B: Cladocera = $24W_{col}-0.05S_{cat}+4.97W_{fl}-10452NH_4-1097NO_3+51P_{min}-47P_{tot}+25381Carb+2543Cl-163TOC-14NTU-6.6HCO_3+215BOD_5-71Cu+859$ (May. $R^2 = 0.9999$); C: Rotifera = $3207(Na+K)-15304Cl+3648SO_4+765Sh_{lakes}+787P_{min}-240P_{tot}-64Mn+42.33W_{col}+3370NO_3-12379BOD_5+253198TDS-26794Pb-28129$ (July. $R^2 = 0.9966$).

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Table 4 shows the most common predictors of 9 regression equations for each of the groups of *Copepoda*, *Rotifera*, and *Cladocera*. The most common predictors of the regression equation for the *Rotifera* number were the catchment basin area, the lake percentage of the catchment basin (6 cases each), the content of sulphates, chlorides, and lead (5 cases each). The following predictors were considered significant ($p < 0.05$): the catchment basin area (6 cases), the lake percentage of the catchment basin, the sulphates content (5 cases). Common and significant ($p < 0.05$) predictors for the numbers of *Cladocera* zooplankters included the catchment basin area (5 cases) and the river discharge (4 cases). The content of chlorides and copper were included in the regression equations at the rate of 4. The numbers of *Copepoda* crustaceans were determined by such parameters of the regression equations as water discharge and the catchment basin area, found at the rate of 6. They were significant ($p < 0.05$) in 6 and 5 cases respectively. The regression equations included such variables as the area of wetlands within the catchment basin, the length of the river and some chemical parameters: content of metals (aluminium, copper and iron), pH, total dissolved solids, content of certain ions (Na+K, NH_4^+, Mg^{2+}), total carbon and total phosphorus, such physical parameters as turbidity and water colour (*Table 4*).

Table 4. *The frequency of inclusion of a predictor in a regression equations (F1) for determining the number of Rotifera, Cladocera, Copepoda and frequency of predictor as significant ($p < 0.05$) (F2).*

Predictor	Rotifera		Cladocera		Copepoda	
	F1	**F2**	**F1**	**F2**	**F1**	**F2**
S_{cat}, km^2	**6**	**6**	**5**	**5**	**6**	**5**
Sh_{lakes}, %	**6**	**5**	-«-	-«-	3	3
Sh_{wetl}, %	-«-	-«-	-«-	-«-	4	4
SO_4^{2-}, mg/l	**5**	**5**	-«-	-«-	4	2
W_{fl}, m^3/s	3	2	**4**	**4**	**6**	**6**
Cl^-, mg/l	**5**	4	**4**	2	3	2
Pb, µg/l	**5**	2	-«-	-«-	3	1
Chl, µg/l	4	4	-«-	-«-	-«-	-«-
Al, µg/l	3	3	3	3	-«-	-«-
TDS, g/l	4	4	-«-	-«-	-«-	-«-
L, km	4	4	-«-	-«-	4	3
P_{tot}, mg/l	4	3	-«-	-«-	4	4
Cu, µg/l	4	2	**4**	2	-«-	-«-
W_{col}, degrees.	3	1	3	3	3	1
Na+K, µg/l	3	3	3	2	3	3
NH_4^+, mg /l	3	3	-«-	-«-	-«-	-«-
Fe, µg/l	3	2	-«-	-«-	3	2
pH	3	2	-«-	-«-	4	2
Turbidity, NTU	-«-	-«-	3	3	-«-	-«-
TOC, mgC/l	-«-	-«-	-«-	-«-	3	3
Mg^{2+}, µg/l	-«-	-«-	-«-	-«-	3	2

-«- – non-inclusion of predictor.

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Discussion

The study performed in 2011–2014 showed that taxonomic composition of the zooplankton community of the rivers of Lake Ladoga local catchment basin and the Neva River was common to the fauna of water bodies of the European North (Kulikova, 2012; Ryabinkina et al., 2012) and was mostly cosmopolitan. The total list of species composition of the zooplankton communities of the studied watercourses, as well as the species richness of most rivers were formed by Cladocera. Rotifers predominated only in two rivers – the Kokkolan and the Burnaya. Virtually all species (99%) were found in Lake Ladoga (Rodionova, 2011). The dominant complex of the river zooplankton communities was formed by the members of lake and littoral macrophyte complexe, due to high lake percentage within the areas of the catchment basins of the studied rivers (up to 14%). Biogeographic analysis shows that it is eurythermic and moderately warm-water species that prevailed in the integrated zooplankton complex of the lake tributaries.

Despite the fact that quantitative parameters of the Ladoga tributaries considerably varied between the stations during the study period, major trends of the zooplankton community development in different seasons can be described. In spring, the number and biomass of zooplankton of large rivers (Svir, Vuoksa, Burnaya, Volkhov, Syas) were essentially formed by the members of Rotifera, while in other tributaries – by copepods, mostly juvenile. In summer, the number of zooplankton in most communities consisted of Copepoda (Cyclopoida) and Cladocera crustaceans, and the biomass – of Copepoda (Cyclopoida and Calanoida) only. In autumn, all the groups of zooplanktons prevailed in numbers in different rivers and in different proportions, but the biomass was essentially determined by the members of Cladocera. The major trends in seasonal changes of the community of crustaceans and rotifers observed at the stations of study were common for lotic communities (Czerniawski et al., 2013; Gromova et al., 2013).

Quantitative parameters of the zooplankton development considerably varied between different stations and different seasons, but the mean values over the period were low, which is typical for the watercourses of the region. The highest values of number and biomass were observed at the station at the Neva River head. *Figures 2* and *3* show that in most rivers mean values of number and biomass of Cladocera crustaceans were higher than mean quantitative parameters of the development of Rotifera and Copepoda zooplanktons. A regular pattern of decrease in quantitative parameters of the zooplankton development in the rivers with smaller catchment basin areas can also be noted (*Figs. 2, 3*). The only exception were low number and biomass at the lower course of a large tributary – the Volkhov River. The zooplankton community of the river mouth can probably be depressed by the Volkhov hydroelectric plant.

The results of the statistical analysis of the data we have performed show that, in case of river clustering based on the species composition and abundance, similar clusters in different seasons of the same year or in different years are almost never formed.

Virtually absent regular patterns in river clustering based on the number of individual zooplankton species and discrepancy in the obtained river clusters and the clusters of similar rivers, based on the physic-chemical parameters and the catchment basins features, indicate high variance and unpredictability of the development of individual species within the Lake Ladoga tributaries.

The results of regression and correlation analyses did not show significant ($p<0.05$) relationship between the parameters of the development of individual zooplankton species and the assessed parameters of water of the Lake Ladoga tributaries.

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Such results prove that abiotic factors and physiographic characteristics of the rivers catchment basins certainly influence the zooplankton in the general way, but apparently the actual structure of community and the populations of individual zooplankton species of the Lake Ladoga tributaries are to a greater extent determined by other factors that were not considered in current study.

Perhaps, these factors include such hydrological parameters as the current speed, the width and depth of rivers, which, according to the concept of river continuum, have great impact on the species composition, the trophic structure, and the distribution of hydrobiont communities (Vannote et al., 1980). The possible influence of the trophic state of the lakes that give rise to rivers and biotic interactions in the water courses on the development of zooplankton communities, should not be excluded, too (Krylov et al., 2007; Czerniawski and Domagała, 2010; Krylov et al., 2011; Chaparro et al., 2015).

Similar results were obtained in case of 54 small water bodies of Wisconsin (USA), showing that the number of species and distribution of spring zooplankton communities were not correlated with physiographic features, pH, electric conductivity, total phosphorus, nitrogen, ions of calcium, sulphates, nitrates, chlorides and silicates (Schell et al., 2001).

In our study, we considered both individual factors that directly influence biota and such integrated variables as catchment basin area, area of wetlands, lake percentage of the catchment basin, etc. These integrated factors virtually determine the parameters considered in our study and the environmental factors that have not been defined and measured. It gives reason to the fact that these integral variables turned out to be the most significant ones and most commonly included in the regression models we obtained (*Table 4*). We did not manage to discover consistent correlations with any of studied parameters of water of the Lake Ladoga tributaries for individual zooplankton species. At the next stage we tried to reveal any possible association between the parameters of the environment and total abundances of Copepoda, Cladocera, and Rotifera groups. We performed multiple forward stepwise regression analysis involving 37 above-listed abiotic parameters at the first stage. The data shown in *Table 4* prove that the integral factors (catchment basin area, water discharge, and lake percentage) are the most significant ones and most often included in the regression equations of the number of Copepoda, Rotifera, and Cladocera.

In earlier studies of the phytoplankton inhabiting the Lake Ladoga tributaries, the dependence of quantitative patterns of the community development on the lake percentage within the rivers catchment basins has also been revealed (Trifonova (ed.), 2006).

The dependence of the structure of plankton communities on the integrated parameters rather than on individual physic-chemical parameters has been shown for different types of aquatic ecosystems. The study of the impact of environmental factors on the zooplankton of lowland rivers of Central Russia has shown that the results of man-induced and zoogenic environmental influence are manifested in different ways, depending on many natural factors, especially hydrological ones (Krylov, 2002a, 2002b). Mechanism of effect of hydrological factors on zooplankton structure and quantitative parameters as element of the lotic ecosystems was shown in (Gromova et al., 2013). The abundance of potamoplankton in the Vistula River (Poland) is also determined by hydrological conditions (Napiórkowski and Napiórkowska, 2013). The variation of water level and the flow pattern are the most important factors of structure and life of alluvial river ecosystems (Junk et al., 1989; Neiff, 1990; Tockner et al., 2000a, 2000b; Bozelli et al., 2015).

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The results of the study of planktonic crustaceans in the reservoir cascade in Brazil indicate that the water residence time (WRT) and such morphometric features of reservoirs as their size, depth, and shape, have a considerable impact on the structure and functioning of zooplankton communities (Bini et al., 2008; Perbiche-Neves and Nogueira, 2010, 2013).

The analysis of the data on the community of crustaceans and rotifers in the regulated Oder River revealed that the most important factor that determines the condition of zooplankton and the physic-chemical properties is WRT (Czerniawski et al., 2013). The influence of hydrochemical factors on the zooplankton of the Novosibirsk reservoir is also connected to the peculiarities of hydrological regime in the parts of the water body (Dvurechenskaya and Yermolaeva, 2014). Zooplankton of 20 lakes of Kejimkujik National Park (Canada) was strongly correlated with such factor as elevation, but not with the studied hydrochemical parameters (Bowman et al., 2014). Only the differences in hydrological and geomorphological conditions led to structural and functional differences in the planktonic communities of the semiarid Macquarie Marshes (Australia) (Kobayashi et al., 2015).

Content of Mg^{2+}, Na^{+} + K^{+} ions and such metals as Pb, Fe, Cu, Al were included in the regression equations less frequently (*Table 4*). It is well-known that depending on chemical and physical speciation, metals can enter the zooplankters in different ways; for instance, dissolved metals can come directly from water, while the particles of associated metals can be consumed with food (Sofyan et al., 2006). Thus, they have different impact on the short-term and long-term changes in structure and composition of the zooplankton community (De Paiva Magalhães et al., 2015). Unfortunately, we have no data on the form of metals present in the water of the tributaries and on their bioavailability for the organisms during the period of our study.

Total phosphorus content was often (up to 4 cases out of 9) included in our multiple regression equations describing total numbers of Copepoda and Rotifera (*Table 4*). Considering low natural concentrations of biogenic elements, especially phosphorus, in the catchment basin of Lake Ladoga (*Appendix A*), one might assume that the concentration of biogenic elements in rivers is the limiting factor for zooplankton, influencing the primary production of phytoplankton. Similar results were obtained for the rivers in Shanghai (China), where population figures and structure of the zooplankton community depended on the content of another biogenic element – total nitrogen, whose concentrations were 20 times lower than those of total phosphorus (Na et al., 2014).

It is interesting that there are many studies describing positive correlation between qualitative patterns of the zooplankton development and water temperature (for instance, in such large rivers as the Danube (Hungary), the Oder (NW Poland)) (Vadadi-Fülöp et al., 2009; Czerniawski et al., 2013), or between it and electric conductivity. However, according to the results of our study, these variables were not included in the equations linking the abundance of zooplankton groups in the Lake Ladoga tributaries and in the Neva River to the environmental factors.

Conclusions

A total of 137 zooplankton taxa ranking below genus were identified in 19 tributaries of Lake Ladoga and the Neva River head in 2011–2014, including 56 species and subspecies of Rotifera (40.9%), 57 – of Cladocera (41.6%), 24 – of Copepoda (17.5%). Quantitative parameters of zooplankton varied in different rivers, still mean values of

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numbers (30–9580 ind./m^3) and biomass (0.17–266.2 mg/m^3) were low. The members of Copepoda and Rotifera were dominant in the lake tributaries in spring, Cladocera – in summer, Cladocera and Copepoda – in autumn.

The development patterns of individual zooplankton species of Copepoda, Cladocera, Rotifera in tributaries of Lake Ladoga was proven to be stochastic. Species composition and patterns of qualitative development cannot be linked to any environmental factors or their combination. It is impossible to identify groups of tributaries of Lake Ladoga with similar patterns of zooplankton development basing on the composition and numbers of individual zooplankton species.

River clustering based on hydrochemical, hydrological parameters and catchment basins' features provides an opportunity to distinguish groups of similar rivers that were consistently formed during different years and seasons. Groups of similar rivers were isolated according to their physiographic position. The cases of belonging of some rivers to different clusters in different time periods turned out to be rare.

Total abundances of Copepoda, Cladocera, and Rotifera in the tributaries of Lake Ladoga are consistently linked with certain combinations of studied physiographic parameters and features of the aquatic habitat, which was proved by the possibility to develop highly significant regression models, describing the number of zooplankton groups as a function of a certain set of variables. Integrated physiographic factors, like the catchment basin area, water discharge, and lake percentage of the catchment basin, are the most important in the level of qualitative development of Copepoda, Rotifera, and Cladocera in tributaries of Lake Ladoga.

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APPENDIX

Appendix A. *Hydrochemical parameters in tributaries of Lake Ladoga and Neva river in 2011–2014: pH, turbidity (TDS, g/l), total phosphorus (P_{tot}, mg/l), total nitrogen (N_{tot}, mg/l), sulphates (SO_4^{2-}, mg/l), carbonates (HCO_3^-, mg/l), chlorides (Cl, mg/l).*

№	River	pH	TDS, g/l	P_{tot}, mg/l	N_{tot}, mg/l	SO_4^{2-}, mg/l	HCO_3^-, mg/l	Cl^-, mg/l
1	Neva	7,39 (0,05)	0,06 (0,04)	0,022 (0,36)	0,59 (0,05)	8,44 (0,16)	30,63 (0,06)	5,48 (0,09)
		6,48-7,60	0,06-0,07	0,012-0,035	0,56-0,64	6,20-9,80	29,30-35,39	4,69-6,24
2	Volkhov	7,27 (0,04)	0,13 (0,15)	0,071 (0,32)	0,93 (0,18)	8,74 (0,27)	71,41 (0,11)	14,39 (0,25)
		6,99-7,89	0,09-0,15	0,038-0,117	0,81-1,26	5,23-12,21	65,17-88,86	9,37-19,14
3	Svir	7,17 (0,04)	0,04 (0,15)	0,024 (0,39)	0,70 (0,12)	5,20 (0,53)	21,42 (0,51)	3,01 (0,26)
		6,34-7,33	0,03-0,05	0,012-0,038	0,60-0,83	3,03-14,36	16,35-56,75	1,78-4,28
4	Syas	7,40 (0,04)	0,11 (0,36)	0,058 (0,24)	0,80 (0,15)	7,31 (0,58)	79,50 (0,44)	5,58 (0,26)
		7,09-7,99	0,07-0,20	0,030-0,078	0,56-0,83	2,06-11,59	48,81-155,50	3,65-7,62
5	Burnaya	7,07 (0,03)	0,05 (0,06)	0,017 (0,67)	0,57 (0,14)	10,72 (0,33)	14,34 (0,08)	4,03 (0,16)
		6,63-7,50	0,04-0,05	0,015-0,068	0,49-0,69	6,63-15,30	12,82-16,35	3,22-5,28
6	Vuoksa	7,09 (0,03)	0,04 (0,15)	24,9 (0,67)	0,69 (0,05)	6,32 (0,11)	20,11 (0,11)	3,57 (0,38)
		6,84-7,57	0,04-0,06	0,020-0,089	0,66-0,76	5,23-7,00	17,82-24,64	2,41-6,70
7	Pasha	6,90 (0,05)	0,05 (0,41)	0,044 (0,43)	0,75 (0,18)	3,89 (0,62)	43,94 (0,56)	3,34 (0,54)
		6,66-7,61	0,04-0,11	0,024-0,078	0,52-0,89	1,92-10,08	20,74-92,50	1,31-6,95
8	Oyat	6,99 (0,05)	0,04 (0,51)	0,048 (0,20)	0,64 (0,13)	3,89 (0,48)	35,07 (0,64)	2,44 (0,47)
		6,48-7,56	0,03-0,10	0,026-0,057	0,48-0,67	1,92-7,01	13,91-84,22	1,23-4,47
9	Yanis	6,73 (0,03)	0,03 (0,09)	0,018 (0,57)	0,62 (0,17)	5,22 (0,10)	6,71 (0,20)	1,97 (0,43)
		6,38-6,87	0,02-0,03	0,011-0,052	0,56-0,81	4,85-6,40	5,37-9,76	1,42-3,83

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№	River	pH	TDS, g/l	P_{tot}, mg/l	N_{tot}, mg/l	SO_4^{2-}, mg/l	HCO_3^-, mg/l	Cl^-, mg/l
10	Olonka	6,60 (0,05)	0,04 (0,28)	96,0 (0,24)	0,74 (0,18)	3,10 (0,39)	18,86 (0,47)	4,47 (0,44)
		6,27-7,20	0,03-0,06	0,064-0,131	0,54-0,87	2,16-6,10	9,30-33,19	2,19-8,54
11	Tulema	6,75 (0,04)	0,02 (0,41)	0,028 (0,78)	0,73 (0,23)	2,75 (0,45)	9,28 (0,48)	1,83 (0,46)
		6,11-7,05	0,02-0,05	0,012-0,099	0,48-0,95	1,92-6,40	3,60-19,28	1,37-3,76
12	Tohma	6,76 (0,04)	0,05 (0,12)	0,034 (0,61)	0,78 (0,05)	10,02 (0,44)	17,09 (0,29)	3,65 (0,68)
		6,44-7,24	0,04-0,05	0,018-0,087	0,73-0,80	4,95-14,65	11,22-25,14	2,91-11,34
13	Kokkalan	7,17 (0,05)	0,05 (0,28)	0,052 (0,61)	0,66 (0,06)	9,71 (0,50)	22,89 (0,35)	4,29 (0,38)
		6,35-7,38	0,03-0,07	0,015-0,096	0,63-0,71	3,36-17,00	9,76-31,00	1,10-5,35
14	Vidlitsa	6,94 (0,03)	0,03 (0,17)	0,044 (0,27)	0,69 (0,17)	3,01 (0,39)	14,59 (0,29)	2,22 (0,44)
		6,54-7,19	0,02-0,04	0,028-0,058	0,54-0,84	2,36-6,10	11,71-23,92	1,37-4,40
15	Uksun	6,40 (0,05)	0,02 (0,24)	0,018 (0,91)	0,59 (0,29)	2,74 (0,28)	3,91 (0,79)	1,58 (0,65)
		5,95-6,82	0,02-0,03	0,012-0,076	0,37-0,68	1,82-3,80	0,73-8,79	1,03-4,54
16	Tuloksa	6,60 (0,07)	0,02 (0,30)	0,086 (0,23)	0,80 (0,13)	2,79 (0,37)	12,57 (0,56)	2,04 (0,39)
		5,81-7,20	0,02-0,04	0,065-0,121	0,65-0,89	1,56-4,90	4,19-22,70	1,37-4,08
17	Lava	7,75 (0,02)	0,17 (0,21)	0,081 (0,31)	1,74 (0,27)	7,35 (0,16)	134,37 (0,17)	9,43 (0,32)
		7,42-7,98	0,15-0,26	0,048-0,108	1,10-1,94	6,05-9,69	121,79-185,93	8,76-17,65
18	Morje	6,59 (0,04)	0,04 (0,30)	0,136 (0,30)	1,28 (0,06)	4,95 (0,29)	12,93 (0,38)	5,82(0,21)
		5,97-6,75	0,03-0,06	0,078-0,186	1,19-1,36	2,55-7,51	10,38-25,63	4,04-7,62
19	Avloga	7,06 (0,04)	0,12 (0,10)	0,293 (0,30)	1,42 (0,52)	12,27 (0,23)	58,80 (0,20)	10,89 (0,12)
		6,90-7,57	0,10-0,14	0,180-0,482	0,91-3,12	7,31-14,36	35,40-70,55	9,50-13,44
20	Nasiya	7,43 (0,02)	0,13 (0,78)	0,089 (0,26)	1,72 (0,47)	6,60 (0,44)	105,88 (0,26)	4,66 (0,23)
		7,20-7,70	0,09-0,51	0,065-0,146	0,83-2,42	4,61-14,21	64,66-141,59	3,01-5,59

Note: Above the line – the median, in brackets – the coefficient of variation, below the line – minimum–maximum values.

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Appendix B. *The number of species of main groups of zooplankton and the dominant species in 2011–2014 in the tributaries of Lake Ladoga and the Neva river.*

№	River	Number of species	Rotifera	Clado-cera	Cope-poda	Dominant species
1	Neva	45	15	19	11	*Asplanchna priodonta* Gosse, 1850, *Euchlanis lyra* Hudson, 1886, *Kellicottia longispina* (Kellicott, 1879), *Notholca caudata* Carlin, 1943, *Bosmina (Bosmina) longirostris* (O.F. Müller, 1785), *Bosmina (Eubosmina) coregoni* Baird, 1857, *Mesocyclops leuckarti* (Claus, 1857), *Eudiaptomus gracilis* (Sars, 1863), *Eudiaptomus graciloides* (Lilljeborg, 1888), *Eurytemora lacustris* (Poppe, 1887)
2	Volkhov	49	15	25	9	*K. longispina*, *Keratella quadrata quadrata* (O.F. Müller, 1786), *B. (E.) coregoni*, *Daphnia (Daphnia) cristata* Sars, 1862, *Disparalona rostrata* (Koch, 1841), *Limnosida frontosa* Sars, 1862, *Scapholeberis mucronata* (O.F. Müller, 1776), *M. leuckarti*, *Thermocyclops crassus* (Fischer, 1853), *E. gracilis*, *E. graciloides*
3	Svir	45	13	21	11	*Asplanchna henrietta* Langhans, 1906, *A. priodonta, E. lyra*, *K. longispina, N. caudata*, *Acroperus harpae* (Baird, 1834), *Alonella nana* (Baird, 1850), *B. (B.) longirostris*, *B. (E.) coregoni*, *Chydorus sphaericus* (O.F. Müller, 1785), *Chydorus ovalis* Kurz,1875, *D. (D.) cristata*, *Polyphemus pediculus* (Linnaeus, 1761), *E. gracilis*, *Limnocalanus macrurus* Sars, 1863
4	Syas	44	13	26	5	*A. priodonta, Cephalodella auriculata* (O. F. Müller, 1773), *Euchlanis dilatata* Ehrenberg, 1832, *E. lyra*, *Synchaeta sp.*, *Alona costata* Sars, 1962, *B. (B.) longirostris, B. (E.) coregoni*, *C. sphaericus*, *Ceriodaphnia quadrangula* (O.F. Müller, 1785), *Oxyurella tenuicaudis* (Sars, 1862), *Pleuroxus truncatus* (O.F. Müller, 1785), *Sida crystallina* (O.F. Müller, 1776), *Eucyclops macrurus* (Sars, 1863)
5	Burnaya	51	24	16	11	*A. henrietta*, *A. priodonta*, *Bipalpus hudsoni* (Imhof, 1891) *K. longispina, Synchaeta sp.*, *C. sphaericus*, *B. (E.) coregoni*, *D. (D.) cristata*, *Microcyclops varicans* (Sars, 1863), *E. graciloides*, *E. gracilis*
6	Vuoksa	57	20	25	12	*Asplanchna herricki* Guerne, 1888, *A. priodonta*, *K. longispina*, *K. q. quadrata*, *C. sphaericus*, *D. (D.) cristata*, *P. truncatus*, *E. gracilis, E. graciloides*, *L. macrurus*

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№	River	Number of species	Rotifera	Clado-cera	Cope-poda	Dominant species
7	Pasha	36	8	23	5	*Euchlanis incisa* Carlin, 1939, *K. longispina*, *A. harpae*, *A. nana*, *B. (E.) coregoni*, *C. ovalis*, *C. sphaericus*, *Graptoleberis testudinaria* (Fischer, 1851), *S. mucronata, E. macrurus*
8	Oyat	39	9	22	8	*A. priodonta*, *E. dilatata*, *B. (B.) longirostris*, *B. (E.) coregoni*, *C. ovalis*, *C. sphaericus*, *P. truncatus*, *Thermocyclops oithonoides* (Sars, 1863), *Eu.gracilis*
9	Yanis	45	7	31	7	*Conochilus hippocrepis* (Schrank, 1803), *Conochilus unicornis* Rousselet, 1892, *E. lyra*, *K. longispina*, *A. harpae*, *B. (B.) longirostris*, *B. (E.) coregoni*, *C. quadrangula*, *C. sphaericus*, *Diaphanosoma brachyurum* (Liévin, 1848), *S. mucronata*, *S. crystallina*, *E. macrurus*, *M. leuckarti*
10	Olonka	43	11	24	8	*E. dilatata*, *E. incisa*, *Synchaeta pectinata* Ehrenberg, 1832, *A. harpae*, *A. nana*, *B. (B.) longirostris*, *B. (E.) coregoni*, *C. quadrangula*, *C. ovalis*, *C. sphaericus*, *Pleuroxus trigonellus* (O.F. Müller, 1785), *P. pediculus*, *S. mucronata, M. leuckarti*
11	Tulema	49	13	26	10	*A. priodonta*, *B. hudsoni*, *E. incisa*, *K. longispina*, *A. harpae*, *A. affinis*, *Alonella exigua* (Lilljeborg, 1901), *A. nana*, *B. (B.) longirostris*, *B. (E.) coregoni*, *C. sphaericus*, *D. brachyurum*, *D. rostrata*, *P. pediculus*, *Cyclops kolensis* Lilljeborg, 1901, *M. leuckarti*
12	Tohma	38	13	18	7	*A. priodonta*, *K. longispina*, *A. harpae, Alona quadrangularis* (O.F. Müller, 1875), *B. (B.) longirostris*, *B. (E.) coregoni*, *C. quadrangula*, *C. sphaericus*, *D. rostrata*, *G. testudinaria*, *O. tenuicaudis*, *Picripleuroxus laevis* (Sars, 1862), *S. mucronata*, *Simocephalus vetulus* (O.F. Müller, 1776), *Eucyclops macruroides* (Lilljeborg, 1901), *Paracyclops poppei* (Rehberg, 1880)
13	Kokka-lan	26	10	9	7	*Ascomorpha sp.*, *A. priodonta*, *E. dilatata*, *K. longispina*, *Keratella quadrata* (O.F. Müller, 1785), *Lecane luna luna* (O.F. Müller, 1776), *B. (B.) longirostris*, *B. (E.) coregoni, D. (D.) cristata*, *D. brachyurum*
14	Vidlitsa	27	11	12	4	*Ascomorpha sp.*, *E. lyra*, *Filinia terminalis* (Plate, 1886), *K. longispina*, *Polyarthra major* Burckhardt, 1900, *Trichocerca elongata* (Gosse, 1886), *A. harpae*, *B. (E.) coregoni*, *C. sphaericus*, *T. oithonoides*, *Cyclops strenuus* Fischer, 1851, *Paracyclops fimbriatus* (Fischer, 1853)

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№	**River**	**Number of species**	**Rotifera**	**Clado-cera**	**Cope-poda**	**Dominant species**
15	Uksun	40	10	25	5	*E. lyra K. longispina, Synchaeta sp.*, *A. harpae*, *A. exigua*, *A. nana, B. (B.) longirostris*, *B. (E.) coregoni, C. quadrangula*, *Chydorus gibbus* Sars, 1891, *C. sphaericus, D. rostrata*, *Eurycercus (Eurycercus) lamellatus* (O.F. Müller, 1776), *Monospilus dispar* Sars, 1862*, P. pediculus, Eucyclops serrulatus* (Fischer, 1851), *T. oithonoides*
16	Tuloksa	46	15	25	6	*E. lyra*, *B. hudsoni, Kellicottia bostoniensis (Rousselet, 1908), S. pectinata, Synchaeta sp.*, *A. harpae*, *B. (B.) longirostris*, *B. (E.) coregoni*, *Ceriodaphnia laticaudata* P.E. Müller, 1867, *C. sphaericus*, *Daphnia (Daphnia) longispina* O.F. Müller, 1785, *D. brachyurum*, *P. trigonellus*, *P. pediculus*
17	Lava	29	9	13	7	*Ascomorpha sp.*, *A. priodonta*, *K. quadrata*, *A. quadrangularis*, *Alona rectangula* Sars, 1862, *B. (E.) coregoni*, *C. sphaericus, P. pediculus*, *S. mucronata*, *Diacyclops languidus* (Sars, 1863), *Macrocyclops albidus* (Jurine, 1820), *P. fimbriatus*
18	Morje	41	14	20	7	*A. priodonta*, *A. henrietta*, *E. insica*, *E. lyra*, *Synchaeta sp.*, *A. quadrangularis*, *A. affinis*, *A. nana*, *B. (B.) longirostris*, *B. (E.) coregoni*, *C.(C.) quadrangula*, *C. sphaericus*, *D. (D.) cristata*, *D. rostrata*, *Rhynchotalona falcata* (Sars, 1862)
19	Avloga	33	12	16	5	*Brachionus calyciflorus calyciflorus* Pallas, 1776, *C. auriculata*, *Filinia longiseta* (Ehrenberg, 1834), *K. longispina*, *Keratella cochlearis cochlearis* (Gosse, 1851), *K. g. guadrata*, *B. (E.) coregoni*, *C. ovalis*, *D. (D.) cristata*, *Daphnia (Daphnia) pulex* Leydig, 1860, *Ilyocryptus sordidus* (Lievin, 1848), *G. testudinaria*, *S. vetulus*, *E. serrulatus*, *M. leuckarti*
20	Nasiya	27	1	21	5	*A. harpae*, *A. affinis*, *A. quadrangularis*, *B. (E.) coregoni, Ceriodaphnia megops* Sars, 1862, *C. sphaericus*, *D. rostrata, P. pediculus*, *S. mucronata*, *E. macrurus*, *E. serrulatus*, *M. leuckarti*, *M. varicans*
Total		137	56	57	24	

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THE REMNANTS OF MID-FOREST IRON ORE EXCAVATIONS AS A REFUGE FOR LOCAL DIVERSITY IN THE VASCLUAR PLANT FLORA

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Abstract. Former excavation remnants can play a significant role in increasing the species richness and local biodiversity on heavily altered, post-industrial sites. In this article we examined 30 plots – mounds and areas surrounding mounds to evaluate the influence of different key factors on floristic composition, including selected edaphic parameters (pH, EC, organic matter, $CaCO_3$ and content of metals: Pb, Cd, Zn, Fe). We also tested the species-area correlations. Additionally, we assessed basic characteristics of plant species, which inhabit those plots, measuring species richness and biodiversity with Shannon-Weiner index (H′) and also estimating the similarities in species composition between mounds and the surrounding areas, using Sörensen's index (S_o). In result, we noted 92 vascular plant species, with mostly native origin (98%). Based on the performed analysis, mounds which were studied had slightly higher values of mean species richness and species diversity compared to the surrounding area. Contribution of socio-ecological groups showed, that, mounds were inhabited predominantly by forest species from *Querco-Fagetea* and *Vaccinio-Piceetea* classes. Analyses of collected soil samples indicated strong influence of pH and content of metals (mostly zinc, lead and iron) in relation to the floristic composition on the examined plots. Unique characteristics of soil samples taken from the studied plots can explain the distinct preferences of some plant species which grow on them. This allowed to classify those structures as a refuge area, considering their positive contribution to the increase of species richness and biodiversity. Nowadays the old remnants can be also treated as a crucial part of historical and cultural heritage.
Keywords: *habitat islands, species richness, human disturbance, local biodiversity, abiotic characteristics*

Introduction

A variety of landscape modifications, which relate to anthropological use and particularly, human destruction of natural habitats, can influence and increase the process of habitat fragmentation, directly threatening the natural state of balance in local diversity (Andrén, 1997). Despite that,, many forms of remnants such as excavations, pits or tailings positively influence species composition during the long term process of reclamation, which occur on them. Man-made sites can also become local shelters for many endangered plant species (Rehounková and Prach, 2006; Rostański, 2005, 2006; Bzdon, 2008; Czylok and Szymczyk, 2009). Due to their distinctive characteristics and role in the process of landscape fragmentation, these structures can be treated as small habitat islands, which corresponds with the main concept of biogeographical theory proposed by MacArthur and Wilson (1967). The state of equilibrium, according to this theory, can be achieved, depending on few basic factors, such as the immigration and extinction rate of plant species, size of studied "island" and level of its isolation from similar structures. In the case of remnants of past activities, we have to consider

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numerous amount of human-based disturbances in form of mining and excavating among the crucial factors, which characterize those differences. In this article we attempt to make a detailed studies on the remnants of mid forest iron ore excavations as a refuge area for local diversity of vascular plant flora, emphasizing the influence of heavily modified abiotic and biotic parameters. We would also like to verify whether other factors (such as the size of the mounds studied) can affect the state of local biodiversity, in accordance with the biogeographical theory of habitat islands.

Materials and methods

Study area

The fieldwork area is located in the Forest District of Koszęcin (Boronów Forestry – Katowice directorate: 118g, 118c, 118a, 117h, 117i, 117g, 117d, 98c and 98i). Fifteen mounds (remnants of iron ore excavation) were investigated during this project. Records were made of vascular plant species composition on those structures, as well as on their surrounding area *(Fig. 1)*, located in the vicinity of two settlements Boronów and Zumpy (Silesian voivodeship). Most of them were already buried and covered by surface layer of soil. Some of the shafts investigated were flooded and then transformed into small artificial reservoirs of rainwater (*Fig. 2*). Every mound was thoroughly measured and their basic parameters were taken, including: length, width, area, height (max and min), the diameter of internal shafts, located on top of the mounds, type of forest habitat and also the GPS coordinates (*Tab. 1*)

Table 1. *Characteristics of the mounds (based on own data)*

No.	Length of mound [m]	Width of mound [m]	Area [m2]	Height (mean) [m]	Height (max) [m]	Diameter of shaft [m]	Type of forest habitat	Longitude	Latitude
1	35	25	875	2.5	3	1.6	MFF	50.69694	18.8998
2	25	24	600	2.2	2.9	2	FCF	50.69682	18.90066
3	26	25	650	3.5	4	6	MFF	50.69738	18.89981
4	30	25.5	765	3	3.3	5	FCF	50.69737	18.90073
5	35.5	17.5	621.25	3.5	3.8	1.2	MFF	50.69807	18.89973
6	26	23	598	5.5	8.5	5	FCF	50.69837	18.89893
7	34	28	952	2.8	3	1.8	FCF	50.69787	18.90064
8	26	26	676	3	3	2	FCF	50.69854	18.90053
9	34.5	31	1069.5	3.5	3.7	2	FCF	50.69858	18.89992
10	28	27	756	2	3	3	MFF	50.69595	18.90024
11	30	25	750	2	2	2	MCF	50.69909	18.90058
12	27	24	648	2	4	5	MCF	50.69959	18.90064
13	24.5	21	514.5	1.8	4	1.5	MCF	50.69909	18.89985
14	24.5	19	465.5	3	4.5	4	MCF	50.69915	18.89893
15	28.5	24.5	698.25	5.5	6	5	WCF	50.69967	18.89976

MFF – mixed fresh forest, **MCF** – mixed coniferous forest, **WCF** – wet coniferous forest

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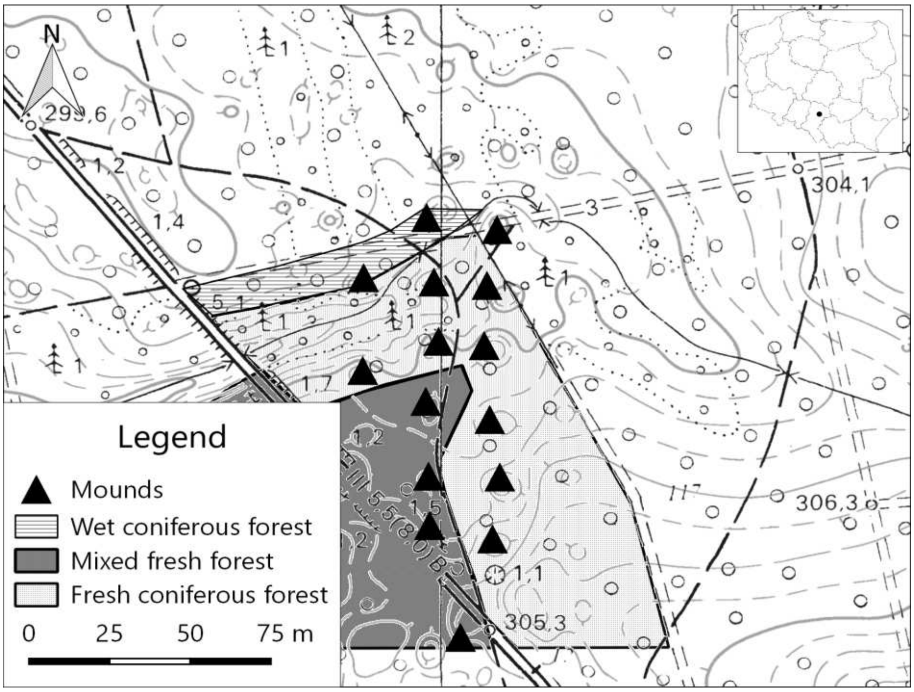

Figure 1. *Localities of excavation remnants – mounds (source: http://www.geoportal.gov.pl, modified)*

Figure 2. *Sample picture illustrating mound and surrounding area (photo A. Rostański)*

Climate and geological conditions

The area of our studies corresponds with the climatic region of western part of the Lesser Poland voivodship, according to the division proposed by Woś (1999). This

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region is characterized by considerably high amount of annual rainwater, due to its specific features, which are typical on the upland site. The mean values of annual temperature set at around 7-8 ° C (the peak is around 14° C in July and the lowest value is -4° C in January). The monthly mean values of rainfall water hover at around 80 mm/m^2 in July and drop to the 40 mm/m^2 in the following months (*Fig. 3*). The winds are mainly western with relatively low speed.

Land relief has slightly sinuous profile with small elevations reaching up to the several meters above ground. In terms of soil conditions, there are mainly 2 types of soils distinguished in this area: proper fallow soils (mostly occurring in the directorates 117 and 118 g) and the proper pseudogley soils (occurring in the directorates 118 c, a and 98 c) (Operat siedliskowy, 2003).

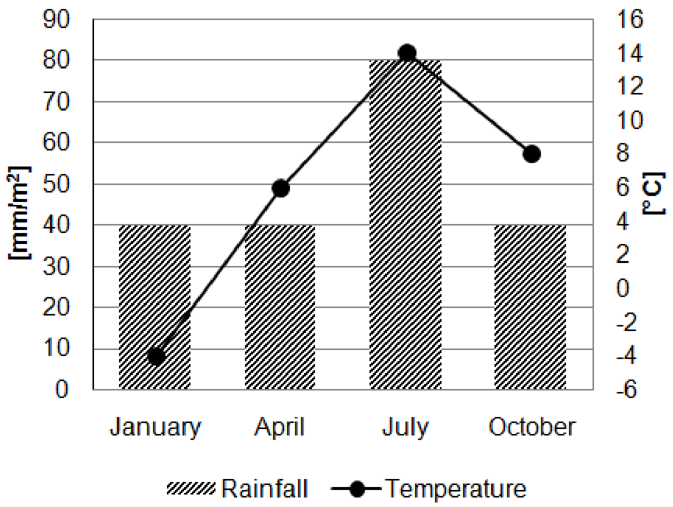

Figure 3. *Climate diagram of area studied with the temperature and rainfall mean values in selected months (source: Kruczała, 2000, modified)*

Methods of fieldwork sampling

For the investigation we established 30 assessment plots, which were differentiated into the excavation mounds themselves (M) and the strip of land around each mound (15 metre in diameter), which was referred to as the surrounding area (S). We used the standard Braun-Blanquet method (1928) to assess cover and constancy for each individual species in the relevés with modified scale (*Tab. 2*). Within those plots, 30 soil samples from depth of 10-15 cm (root zone) were taken (bulk samples from each mound and surrounding area) and prepared for the laboratory analysis.

Table 2. *Constancy scale for species noted*

Degree	% constancy
V	100-80
IV	80-60,1
III	60-40,1
II	40-20,1
I	20-1

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Methods of biotic analyses

The nomenclature for plant species was taken from Mirek et al. (2002). Their origin (including apophytes – native species occurring on synanthropic habitats and kenophytes – species permanently established in Poland after the year of 1500) was based on classifications from Tokarska-Guzik (2005) and Mirek et al. (2002). Affinity to socio-ecological groups of designated plant communities was assigned, based on classification of Matuszkiewicz (2007) with distinction of 6 classes, which had the highest contribution: *Querco-Fagetea* and *Vaccinio-Piceetea* (forest communities), *Molinio-Arrhenatheretea* and *Epilobietea angustifolii* (wet meadows and nitrophilous communities), *Nardo-Callunetea* (turfs and moors communities) and *Artemisietea vulgaris* (ruderal communities of perennials). Relevant species traits, including life strategies were estimated, based on Grime (1979, 2002) and Klotz et al. (2002) classifications, which allowed primary strategies (C – competitors, R – ruderals, S – stress tolerators) to be distinguished, as well as some of the mixed strategies (CR – composition/ruderal, CS – competitors/stress tolerators, SR – stress tolerators/ruderals and CSR – competitors/stress tolerators/ruderals).

Vegetation diversity and similarity indices

The level of overall biodiversity within plots was measured using the Shannon-Wiener index of diversity (H'), based on the species cover values noted (Whittaker 1972), according to this formula:

$$H' = -\sum_{i=1}^{n} Pi \ln Pi \quad \text{(Eq. 1)}$$

Pi – number of individuals of i-th species
n – total number of species

In order to measure the similarity level in species composition between mounds and surrounding area plots, Sörensen index of similarity (S_o) was applied (Trojan, 1980), as the necessary calculations were made according to this formula:

$$So = \frac{2c}{a+b} \times 100\% \quad \text{(Eq. 2)}$$

where **a** represents the number of species on the mounds, **b** represents the number of species in the surrounding area and **c** represents the number of species which are common for both facilities. This index shows the resemblance on the percentage scale, where 0% means lack of any common species between specific mound and its surrounding area and 100% means that, species composition in both cases is identical.

Methods of soil sample analysis

The soil samples collected from the plots were dried at 105 °C and sieved through 2mm mesh. Active pH (in H_2O) and electric conductivity (EC) were measured using a pH/conductivity/TDS meter. The content of organic matter (% OM), was measured, based on the loss of ignition analysis of dried samples. Percentage equivalent of the calcium carbonate (% Eq$CaCO_3$), was estimated using Scheibler's method. Metal

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concentrations (lead, cadmium, zinc and iron) in the soil samples were analyzed using the flame atomic absorption spectrometry (AAS).

Statistical methods and correlations

All of the listed parameters, which refer to the vegetation (diversity index and number of species noted) and soil (pH, electric conductivity, % Eq$CaCO_3$, content of organic matter, content of metals) properties have been also measured, to identify statistical differences in pairs of plots (mound-surrounding area) using standard U-Mann Whitney test. For the correlation in species diversity-area model we transformed data using semi-log scale and used linear regression method and also testing the statistical significance. We applied the canonical correspondence analysis (CCA) to estimate the species-environmental relations and we also used the cluster analysis (Ward method with Euclidean distance) to indicate whether the physico-chemical variables are distinctive for each group of plots. The following software was used to perform the statistical calculations and visual interpratations: CANOCO 4.5 (Ter Braak and Šmilauer, 2002; Lepš and Šmilauer, 2003) Statistica 12 (StatSoft 2011) and RStudio 1.01 (2015).

Results

Vegetation diversity and similarity measurements

There were 92 vascular plant species noted together on the mounds and their surrounding area (*Appendix 1*). Based on the mean values of the species diversity indices we observed that, the mounds had slightly higher species richness compared with the area which surrounded them (23 ± 2.21 on mounds and 19 ± 2,13 in the surrounding area), following the slightly higher values of species biodiversity noted (4.31 ± 0.15 on mounds and 4.01 ± 0,15 in the surrounding area), although that, difference between those groups was estimated as not statistically significant according to the results of conducted U-Mann Whitney test (*Tab. 3*).

Table 3. *Mean values (with standard error) for biotic parameters and results of the U-Mann Whitney test (* – significance at p<0.001)*

Parameters	Mounds	Sur. area	Z	p
Number of species	23 ± 2.21	19 ± 2,13	1.267339	0.205035
Diversity index (H′)	4.31 ± 0.15	4.01 ± 0,15	1.244342	0.213375

Native species (98%) were almost exclusively building the composition of the flora investigated, which is a commonly observed phenomenon on abandoned areas with post industrial origin. Only 2% of the total composition was represented by alien species (kenophytes), such as: *Impatiens parviflora* (mainly concentrated in the surrounding area) and *Quercus rubra*, (recorded on one of the studied mounds).

There were 13 different socio-ecological classes distinguished in the plots sampled, although some of them were represented only by single species. Most of the mid-forest mounds consisted of typical forest and scrub species (representing the classes: *Querco-Fagetea* and *Vaccinio-Piceetea*). Together they exceeded a contribution of 38%. The second and also influential group, consisted of fresh meadows species (representing the

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class: *Molinio-Arrhenatheretea*) (*Fig. 4*), with a contribution level of 18%. If the results are compared the in three different groups of species, representing those which occurred only on mounds (M), those which occurred on mounds and in the surrounding area (M/S) and those which occurred only in the surrounding area (S), we observed a higher contribution of species from *Querco-Fagetea* and *Artemisietea vulgaris* classes, which were present only on mounds, compared with those species, which exclusively inhabit the surrounding area (*Fig. 5*).

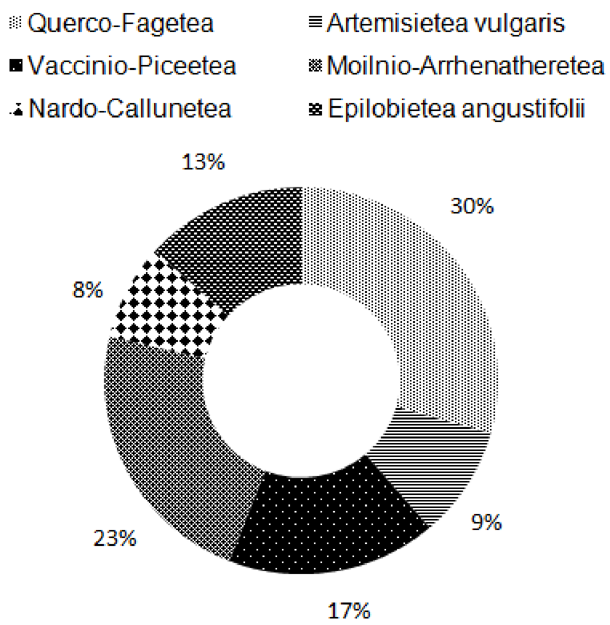

Figure 4. *Contribution of the most numerous socio-ecological classes (based on total flora)*

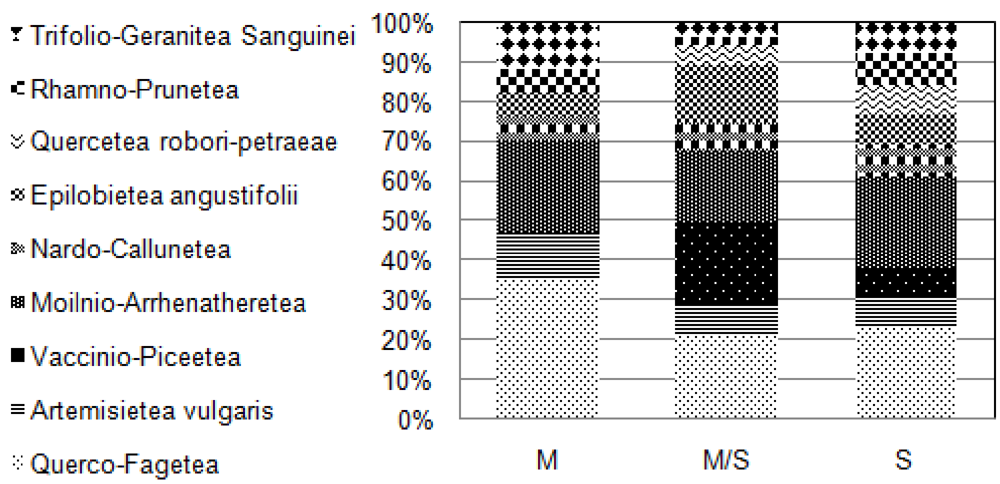

Figure 5. *Contribution of socio-ecological classes based on the species occurrence (only on mounds – M, on mounds and* surrounding *area – M/S, only in the surrounding area – S)*

These mid-forest excavating mounds and the areas immediately around them were predominantly inhabited by species which represented C (competitors) strategy (33) and

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CSR (competitors/stress tolerators/ruderals) strategy (30) (*Fig. 6*). The contribution of species, representing C and CS strategies was higher on mounds, although surrounding areas had slightly higher contribution of species with CSR type of life strategy.

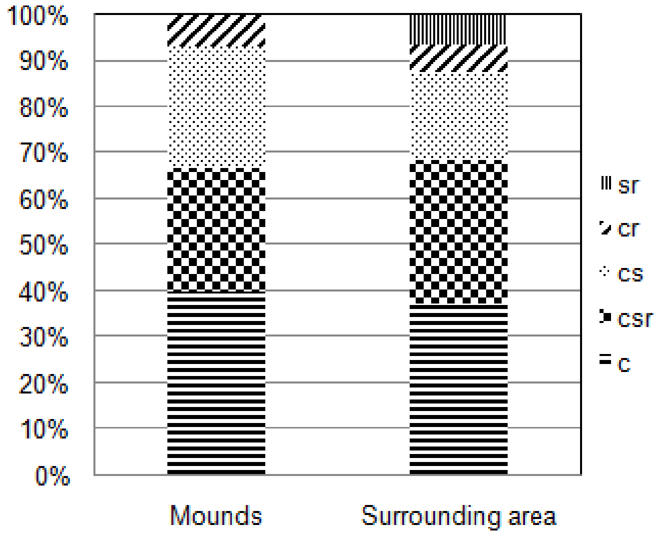

Figure 6. *Contribution of life strategies in both groups of plots*

Most of the species noted belong to constancy class I both on the mounds (12) and in the surrounding area (14), which corresponds with a low amount of individual species and a relatively high contribution of common ones, although a few species were present only on mounds and had substantially high level of constancy there (IV, III and II classes of constancy). In addition, some plant species were present only in the areas around the mounds (II and III classes of constancy) (*Fig. 7* and *Tab. 4*). Rare and valuable plant species, like: *Daphne mezereum* (IV class), *Luzula multiflora* (II class), *Galium schultesii* (II class) represented the general group, whose occurrence was exclusively limited to the mounds. At the opposite extreme, common woody species, such as: *Acer platanoides* (II class) and *Hieracium murorum* (III class) had relatively high frequency level in the surrounding area.

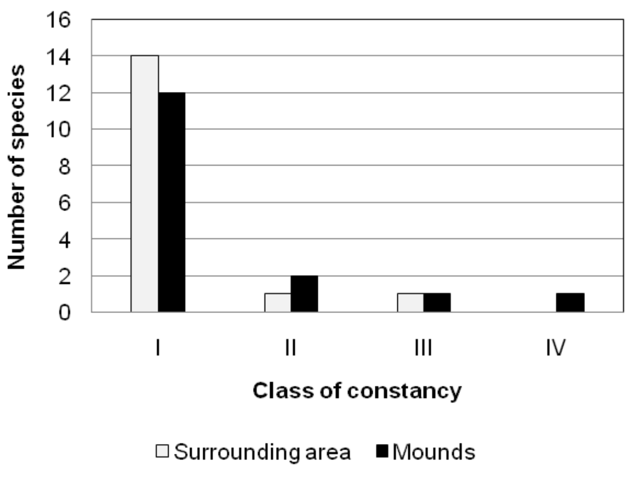

Figure 7. *Number of species in the distinguished classes of constancy*

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Table 4. *Constancy values for the plant species which exclusively appear on mounds or in the surrounding area*

	Species name	I	II	III	IV
Mound plots	*Astragalus glycyphyllos*	•			
	Carex spicata	•			
	Corylus avellana	•			
	Epilobium roseum	•			
	Galium aparine	•			
	Lychnis flos-cuculi	•			
	Melica nutans	•			
	Prunus spinosa	•			
	Senecio ovatus	•			
	Stachys sylvatica	•			
	Scutellaria galericulata	•			
	Vicia sylvatica	•			
	Galium schultesii		•		
	Luzula multiflora		•		
	Lathyrus pratensis			•	
	Daphne mezereum				•
Surrounding area plots	*Agrostis capilaris*	•			
	Calluna vulgaris	•			
	Circaea lutetiana	•			
	Centaurium erythreaea	•			
	Epilobium hirsutum	•			
	Festuca gigantea	•			
	Jasione montana	•			
	Phleum pratense	•			
	Polygonum persicaria	•			
	Potentilla anserina	•			
	Sarothamnus scoparius	•			
	Prunella vulgaris	•			
	Quercus rubra	•			
	Mentha arvensis	•			
	Acer platanoides		•		
	Hieracium murorum			•	

The comparison between mounds and their surrounding areas in regard to their level of similarity highlighted some noticeable discrepancy between studied groups of plots. The highest values of Sörensen coefficient were noted on mounds 14 (S_o= 61.54%) and 15 (S_o= 60%) with 8 and 6 common species. In the contrary, one of the lowest levels of similarity were noted in the plots 9 (S_o= 38.89%) and 13 (S_o= 41.38%) with 7 and 6 common species respectively (*Fig. 8* and *Tab. 5*). Furthermore, one of the biggest mounds (with number 9 and 13) had the lowest value of commonly shared species which indicates their substantial distinctiveness compared to the smaller ones. This also proves the theory, that, those habitats which correspond with the large islands are less prone to the extinction threat with their optimized species pool.

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The relationship between species diversity and area of mounds

We also wanted to examine the linear correlation in the commonly used species-area model, although we put more focus on the investigation of species diversity of local plant communities, which better emphasizes the essentials of our studies. In case of our mounds which have been divided into three separate size categories (*Tab. 6*), the correlation between those variables was determined as positive, although not statistically significant ($r^2 = 0.2923$, $P = 0.021$). According to the previously mentioned theory, relatively small mounds, due to the size limitations, tend to have less diverse species pool, comparing to the bigger ones which, but at the same time, can support more heterogeneous habitats. Moreover, the biggest mound reached its threshold which resulted in much lower species diversity compared to the other structures (*Fig. 9*).

Table 5. *Number of species (mean ± SE) which are present on the plots (commonly, only on the mounds, only in the surrounding area)*

Number of mound	**Number of species present**		**Number of common species**
	Mound	Surrounding area	
1	35	36	19
2	27	32	17
3	27	17	10
4	34	23	13
5	18	19	8
6	12	13	6
7	40	31	16
8	27	23	13
9	21	15	7
10	15	10	6
11	22	11	8
12	20	17	8
13	17	12	6
14	11	9	6
15	13	13	8
Mean ± SE	**23 ± 2.21**	**19 ± 2.13**	**10 ± 1.10**

Table 6. *Species diversity in relation to the different size categories distinguished (H′ – diversity index)*

Categories	**Area [m²]**	**H′**
Small mounds (< 600 m²)	465.5	3.316
	514.5	4.024
	598	3.472
Medium mounds (600-800 m²)	600	4.687
	621.25	4.059
	648	4.262
	650	4.658
	676	4.677

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	698.25	3.683
	750	4.359
	756	3.865
	765	4.999
Large mounds (> 800 m²)	875	5.058
	952	5.266
	1069.5	4.303

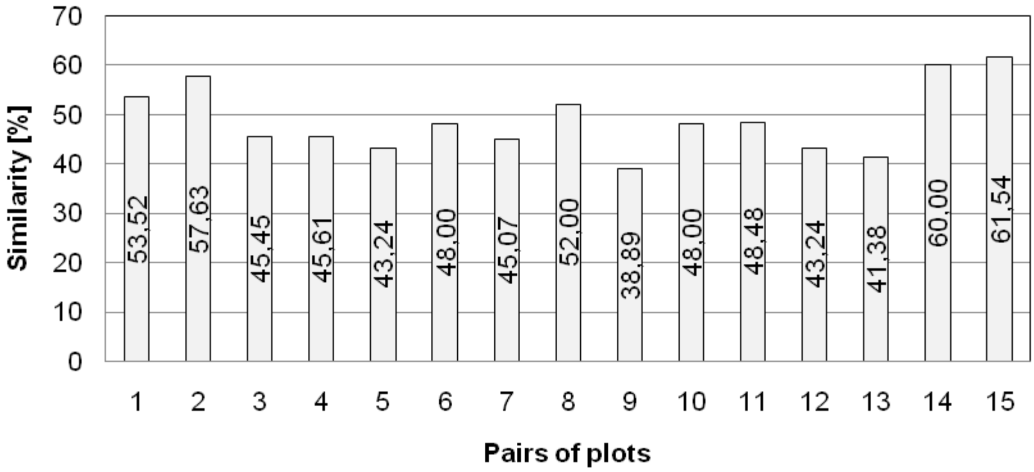

Figure 8. *Sörensen's index of species similarity for pairs of plots (mound-surrounding area)*

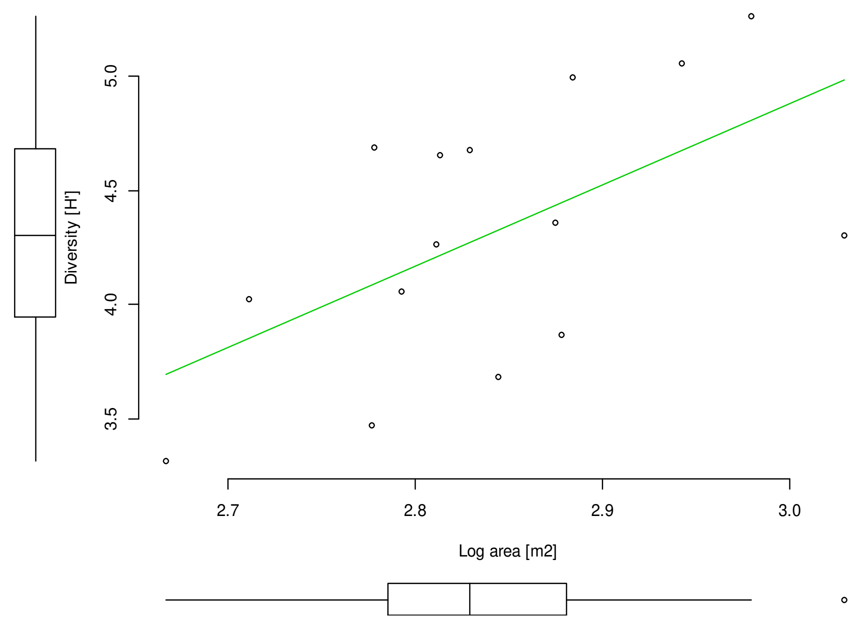

Figure 9. *The relationship between species diversity (H') and size of the mounds (n= 15, y= 3.6x -5.8)*

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Correlation between soil parameters and composition of vascular plant flora

We determined that, there is a difference between soil samples taken from mounds and surrounding areas, specifically related to the variables: pH ($Z = 4.21$; $p = 0.000025$), content of zinc (Zn) ($Z = 4.64$; $p = 0000003$) and iron (Fe) ($Z = 4.64$; $p = 0000003$) with the significance level confirmed by the results of the U-Mann Whitney test (*Tab. 7*). The values of these variables also indicate the main components in the canonical correspondence analysis. The respective scores of canonical correspondence analysis showed, that, the first and second axis describe 13.1% of total variation in the floristic composition (*Tab. 8*).One of the analyzed edaphic factors (content of calcium carbonate (Eq% $CaCO_3$)) was omitted due to its ambiguous correlation to both axes, which caused slight distortion. The right side of the graph (*Fig. 10*) presents a group of correlated variables (pH, Fe and Zn), which corresponds with the first axis positively and also explains the external differentiation between mounds (M) and surrounding area (S). The second, quite substantial group (related to organic matter, electric conductivity and content of Cd and Pb) is correlated with the second axis, which is more likely responsible for the process of internal differentiation within each group of plots (mounds and surrounding areas respectively).

The cluster analysis (Ward method with the Euclidean distance) also highlighted the fact, that, the environmental conditions of studied sites (mounds and surrounding areas) are definitely unique, which manifested in occurance of two major groups of variables and one additional group on the graph (*Fig. 11*). The first group (on the right side) of surrounding area plots (S) is considered less homogenous with two subgroups representing greater distance between them. In contrary, in the second group (in the middle) there was only single plot (13) separating from the main two subgroups, although the distance between those two major subgroups is considerably smaller. The appearcence of mixed group (with surrounding area and mound plots) explains the fact, that, some of the plots were located in the same linear transect.

Table 7. *Mean values (with standard error) for the soil parameters (* – significance measured by the U-Mann Whitney test at p <0.001)*

Parameters	Mounds	Sur. area	Z	p
pH_{H2O}	4.94 ± 0.11	4.09 ± 0.09	4.210492	0.000025*
EC [µS]	106.06 ± 12.43	86.13 ± 9.16	0.871524	0.383469
OM [%]	16.66 ± 1.43	13.06 ± 1.91	1.907991	0.056393
Pb [mg kg^{-1}]	68.55 ± 11.37	62.94 ± 9.79	2.115381	0.034398
Eq$CaCO_3$ [%]	0.15 ± 0.04	0.08 ± 0.02	0.943011	0.345676
Cd [mg kg^{-1}]	1.37 ± 0.29	0.58 ± 0.13	0.248868	0.803463
Zn [mg kg^{-1}]	167.2 ± 12.88	39.05 ± 5.91	4.645544	0.0000003*
Fe [mg kg^{-1}]	52104.03 ± 5513.39	6310.02 ± 1314.98	4.645544	0.0000003*

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Table 8. *Summary results of CCA analysis of plots studied*

Axes:	1	2	3	4
Eigenvalues:	0.305	0.116	0.103	0.086
Species-environment correlations:	0.916	0.817	0.784	0.758
Cumulative percentage variance of species data:	9.5	13.1	16.3	18.9
of species-environment relation:	39.3	54.3	67.5	78.6

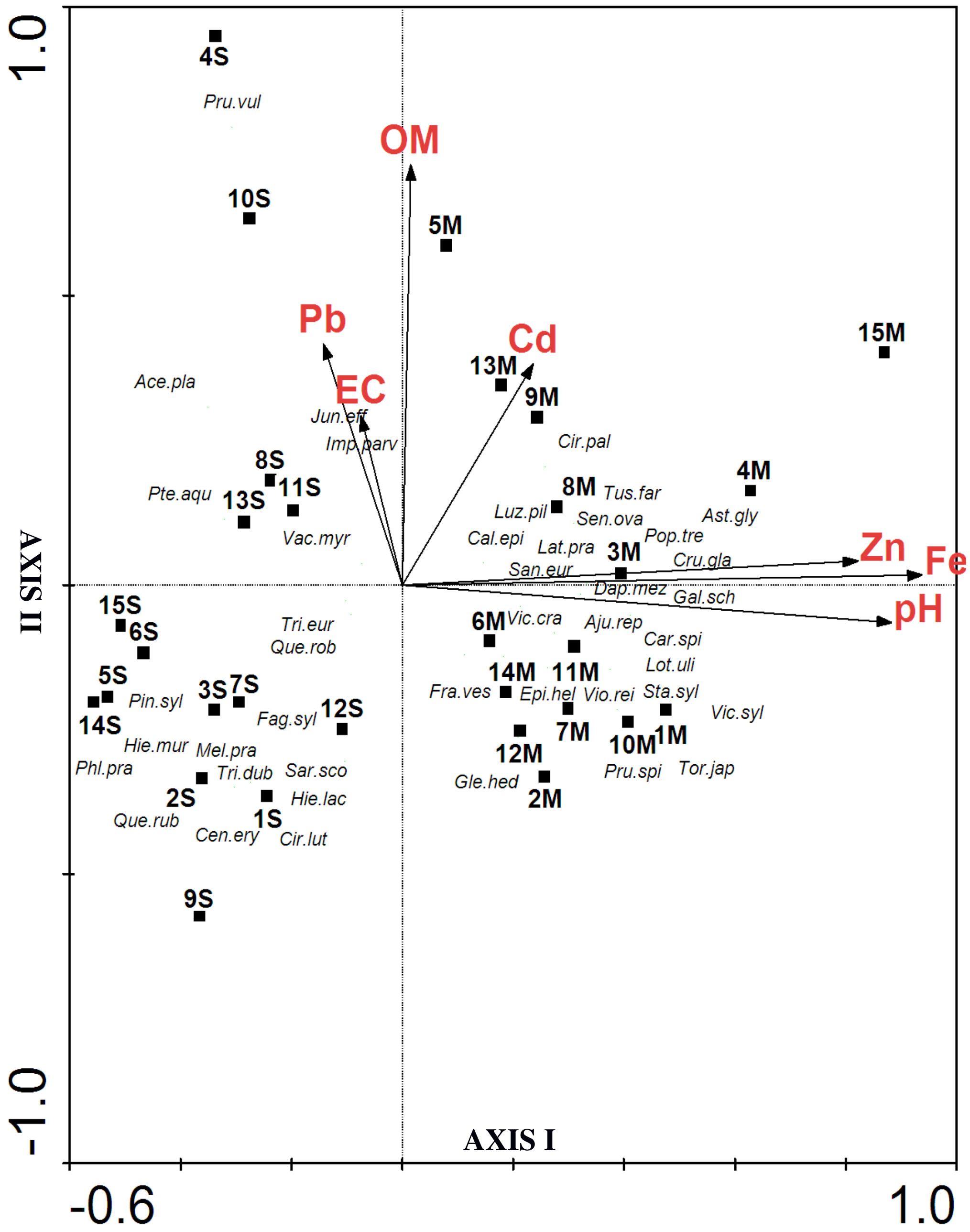

Figure 10. *The relationship between soil properties and the species composition based on the results of CCA analysis (M – mounds, S – surrounding area)*

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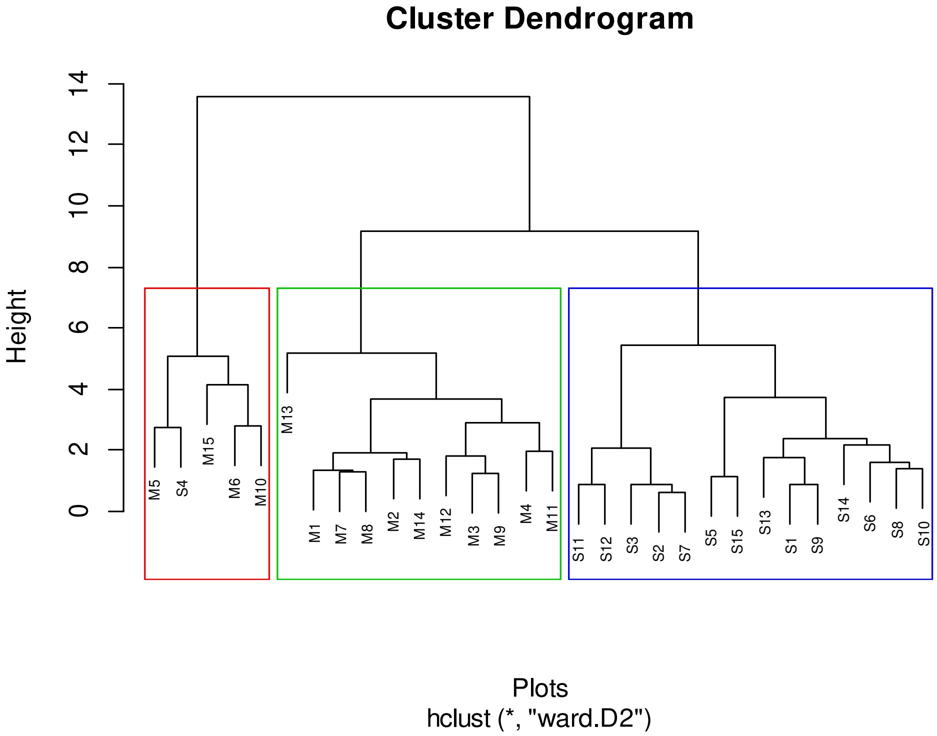

Figure 11. *Cluster analysis (Ward method with Euclidean distance) of the physico-chemical soil properties measured on mound (M) and surrounding area (S) plots*

Discussion

Based on the obtained results of analyses, which included all of previously listed biotic and abiotic variables, the mid-forest mounds are clearly distinguished, from the areas which surrounds them. Further studies showed, that, the floristic composition of plots consisted mainly of native species (apophytes), with a marginal contribution of alien species (kenophytes). Forest communities (from classes: *Querco-Fagetea* and *Vaccinio-Piceetea*) dominated in the composition of the flora in both types of plots studied (mounds and surrounding area), with a slightly higher contribution of species from *Querco-Fagetea* class on the mounds (35 %), than in the surrounding area (22%). The higher values of contribution of species from *Artemisietea-vulgaris* class was noted on the mounds studied. The results of research conducted on the similar structures in the Gielniowski Hump (Małopolska Upland) also confirmed that, mesophilous forest communities, which consist of predominantly native species, can colonize these habitats more frequently. The studies also suggest that, throughout the extensive mining activities, the influence in local habitats could potentially increase the overall species richness (particularly by species from *Querco-Fagetea* class), as well as local diversity (Podgórska, 2010, 2016). During the studies we determined, that, some of the plant species were more commonly distributed on mounds, with their relatively high level of contribution, like *Lathyrus pratensis* or partially protected in Poland species *Daphne mezereum*. Other species like *Acer platanoides* or *Hieracium murorum* had relatively higher constancy in the surrounding area plots. Based on analyses of species distribution in each group of life strategies, the investigated plots were characterized by a high contribution of species representing the C (competitors) and CSR (competitors/stress

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tolerators/ruderals) strategies. It has been shown that, moderate disturbance and stress are factors which can potentially limit the process of competition between plant species on various anthropological habitats. Substantial contribution of plant species with C (competitors) strategy is also expected in places, where both human and natural stress factors (related to mining activities, fires or floods) have ceased to exist for a long time (Nowak, 2009). Sörensen's index of similarity showed that, the majority of mounds have a vast amount of common species, which can also occur in the surrounding area. Although, one of the biggest mound (number 9) had the lowest similarity index which contribute to the assumption that, large habitat islands have their own unique species pool. The results of diversity-area studies, based on the linear regression method, showed positive correlation between species diversity and size of inhabited mound (island), indicating strong relationship between those variables. Species richness is mostly related to the amount of different habitats created on the island. Typically, more spacious islands contain more types of habitats, which directly translates into increase in species richness (Kohn and Walsh, 1994; Rickless and Lovette, 1999). Also, due to the fact that, small islands consist of rather small species pools, different environmental modifications and human related disturbances, even at a relatively low level, can have a strong impact on the composition of the vascular plant flora which occupies them (Panitsa et al., 2006). However, in some individual cases, we can observe the "small island effect", where species diversity is actually independent from the size parameter (Triantis et al., 2006). According to the results of the canonical correlation analysis, the soil variables measured: pH, content of Fe and Zn, were considered as valid, key factors which explain the differences between observed groups (mounds and surrounding areas). Further division between plots have been shown to be influenced by other parameters, including organic matter (OM), electric conductivity (EC) and content of the heavy metals Cd and Pb. High content of heavy metals, alongside other detrimental soil characteristics have been considered as one of the possible factors influencing the rate of colonization process by plant species on iron ore tailings Ma On Shan in the Hong Kong area (Wong and Tam, 1977). A variety of iron tailings and remnants are also characterized by significantly high amount of other heavy metals (including Pb, Zn, Ni and Cu) (Zhang et al., 2006). Some studies also prove that, low pH values can have a strong impact on the composition of plants, representing typical woody communities (Podgórska, 2015; Peet et al., 2003). Some authors also claim that, values of pH between 5-6 are preferable for many plant species, although, when soil become more acidic or alkaline, the number of tolerant species decreases and more specialists start to occur (Schuster and Diekmann, 2003). In contrast, others suggest that, higher concentration of calcium (Ca), which is often correlated with increased values of pH, can possibly have more impact in the process of reclamation on a variety of mine tailings, than the higher uptake of heavy metals (Hossner and Hons, 1992).

Conclusions

The remnants of iron ore excavations undoubtedly play a significant role influencing the state of local diversity which was confirmed by increasing number of species (with native origin), representing typical woody communities. In result of long inactivity of mining process we observed strong contribution of species with C (competitors) life strategies, although species with CSR (competitors/stress tolerators/ruderals) were also significantly present in the composition of the vascular plant flora. During our studies

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we presented strong correlation between modified soil properties and species composition on the mounds investigated. The excavating process, which involves mining lower layers of soil and depositing the material on the surface resulted in completely altered soil parameters on the mounds, which promoted some of plant species to inhabit them, which led to some relevant differences in species composition, compared with the surrounding areas. The composition of vascular plant flora of former excavating sites indicates the process of their gradual naturalization, which manifests through the domination of native species and occurrence of some valuable species. Apart from the natural values of mounds studied, they can be also treated as a part of historical and cultural heritage.

Despite the negative affection of human disturbances on the mounds studied in this project, their appearance significantly improved the level of species richness and local biodiversity of typical forest habitats around Boronów district.

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APPENDIX

***Appendix 1.** List of plant species with their cover(Braun-Blanquet scale), which were noted on the plots (M – mounds, S – surrounding area)*

		M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 13	M 14	M 15	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	S 10	S 11	S 12	S 13	S 14	S 15
ChCl: Agropyretea																															
Equisetum arvense	c	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.
ChCl: Artemisietea vulgaris																															
Epilobium hirsutum	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	.	.
Glechoma hederacea	c	1	1	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
Impatiens parviflora	c	1	2	.	1	.	.	2	.	.	.	.	.	.	.	.	2	1	.	3	.	.	1	1	.	.	.	1	.	.	.
Torilis japonica	c	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Urtica dioica	c	2	.	1	2	.	.	2	.	1	2	.	.	1	.	.	1	2	.	2	.	.	1	2	.	2	.	.	1	.	1
Occasional species: *Epilobium roseum*(+), *Galium aparine*(r)																															
ChCl: Epilobietea angustifolii																															
Sambucus nigra	b	.	1	1	1	.	.	.	1	1	1	.	.	.	.	.	1	.	1	.	.	.	1	.	1	.	.	.	.	.	.
Calamagrostis epigejos	c	1	2	3	4	3	3	3	3	3	.	3	.	.	2	.	2	2	2	2	2	3	1	1	1	.	1	.	.	1	.
Centaurium erythraea	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Fragaria vesca	c	2	2	2	2	1	.	1	1	1	1	2	2	2	.	1	2	3	.	.	.	1	.	.	.	.	.	.	.	.	1
Myosotis sylvatica	c	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
Senecio ovatus	c	.	1	2	.	.	.	.	1	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ChCl: Koelerio glaucae-Corynephoretea canescentis																															
Jasione montana	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.
ChCl: Molinio Arrhenatheretea																															
Campanula patula	c	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	r	r	.	.	.	.	.	.	.	.	.	.	.	.	.
Cirsium palustre	c	.	.	2	r	1	.	.	.	.	.	1	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.

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Deschampsia caespitosa	c	1	1	.	1	1	2	.	1	.	.	1	.	.	.	.	1	.	.	1	1	2	1	.	.	.	1	.	.	.	.
Juncus effusus	c	1	.	1	.	.	.	1	1	.	.	.	.	.	.	.	1	.	.	2	.	.	1	1	.	.	.	1	1	.	.
Lathryus pratensis	c	.	1	.	1	1	.	1	.	1	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lotus uliginosus	c	1	.	1	1	.	.	1	1	1	.	1	1	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.
Lychnis flos-cuculi	c	.	.	.	.	.	.	+	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lysimachia vulgaris	c	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	2	.	1	.	.	.	.	.	.	.	.	.	.
Potentilla anserina	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1	.	.	.
Prunella vulgrais	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.
Ranunculus repens	c	1	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Selinum carvifolia	c	1	1	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
Trifolium dubium	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.
Vicia cracca	c	1	1	.	1	1	.	1	1	.	1	1	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.
Occasional species: *Phleum pratense*(+)																															
ChCl: Nardo-Callunetea																															
Agrostis capillaris	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.
Calluna vulgaris	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	2	.	.	.	.	.	.	.	.	.
Hieracium lachenalii	c	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Luzula multiflora	c	.	.	.	1	.	.	1	2	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Potentilla erecta	c	.	.	.	1	.	.	.	.	.	1	.	.	.	.	1	1	1	1	1	1	.	1	1	1	1	1	.	.	.	1
Veronica officinalis	c	1	.	1	.	.	.	1	.	1	.	.	.	1	1	1	1	.	1	.	.	1	1	.	1	.	.	1	.	.	1
ChCl: Phragmitetea																															
Scutellaria galericulata	c	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ChCl: Quercetea robori-petraeae																															
Hieracium murorum	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	2	.	1	1	1	.	1	.	.	.	.	.	.
ChCl: Querco-Fagetea																															
Acer platanoides	a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.

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Fagus sylvatica	a	.	1	.	.	.	.	.	.	.	.	1	.	.	.	.	1	1	1	.	1	1	.	.	.	.	.	.	.	.	.
Corylus avellana	b	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Daphne mezereum	b	1	1	1	1	1	.	1	.	.	1	1	.	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Actaea spicata	c	.	1	.	1	.	.	.	.	.	.	.	.	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Aegopodium podagraria	c	1	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.
Carex remota	c	.	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.
Carex sylvatica	c	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	1	1	.	.	.	.	1	.	.
Circaea lutetiana	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3	.	2	.	.	.	.	.	1	.	.	.	.	.	.
Dryopteris filix-mas	c	.	.	.	.	.	.	.	.	.	.	.	1	1	.	1	1	1	1	1	.	.	1	1	.	.	.	.	1	.	1
Epipactis helleborine	c	1	.	1	1	.	.	.	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.
Festuca gigantea	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.
Galium schultesii	c	.	.	.	1	.	.	1	1	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Melica nutans	c	.	.	.	.	.	.	.	.	.	.	1	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sanicula europaea	c	2	2	2	2	.	.	2	2	2	2	.	.	2	.	1	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.
Stachys sylvatica	c	2	.	1	.	.	.	2	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Viola reichenbachiana	c	2	2	1	1	.	.	2	2	1	1	.	.	.	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.
Occasional species: *Scrophularia nodosa*(1)																															
ChCl: Rhamno-Prunetea																															
Prunus spinosa	c	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sarothamnus scoparius	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	r	.	.	.	.	.	.	.	.
ChCl: Scheuchzerio-Caricetea nigrae																															
Stellaria palustris	c	.	.	.	2	.	.	.	.	.	2	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	1	.	.
ChCl: Trifolio-Geranietea sanguinei																															
Astragalus glycyphyllos	c	.	.	.	1	.	.	.	.	.	.	.	.	.	r	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Vicia sylvativa	c	2	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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ChCl:Vaccinio-Piceetea																															
Picea abies	a	.	.	.	1	1	.	1	1	1	.	.	.	1	.	1	.	.	.	.	1	.	1	1	1	.	.	.	1	.	1
Pinus sylvestris	a	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	1	.	.	2	2	.	.	1	.	.	.	.	.	.
Dryopteris dilatata	c	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1	.	.	.	1	.	.	1	.	.	.	.	1	.	2	1
Melampyrum pratense	c	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	3	2	.	.	2	2	.	.	.	.	.	.	.	2	.
Trientalis europaea	c	2	1	1	1	.	1	2	1	1	1	.	1	1	1	.	2	1	2	2	2	.	2	1	2	2	2	.	.	.	.
Vaccinium myrtillus	c	2	4	2	.	5	3	2	4	2	.	5	3	.	3	.	3	3	3	4	4	3	3	3	3	4	4	3	1	.	1
Accompanying species																															
Betula pendula	a	.	.	.	1	1	1	1	1	1	.	.	.	.	.	.	.	1	.	.	.	.	1	1	.	.	.	.	.	.	.
Larix decidua	a	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	1	.	.	.	.	.	.
Populus tremula	a	.	.	.	2	.	1	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Quercus robur	a	1	1	.	1	1	1	1	1	.	1	1	1	.	.	.	1	2	1	1	1	1	1	2	1	1	1	1	.	.	.
Frangula alnus	b	.	.	1	.	.	.	.	1	.	.	.	.	.	.	.	1	2	.	1	.	.	.	1	.	.	.	.	.	.	.
Sorbus aucuparia	b	.	1	.	.	.	1	.	1	.	.	.	1	.	.	.	.	1	.	.	.	.	.	1	.	.	.	.	.	.	.
Ajuga reptans	c	1	1	1	1	.	.	1	.	.	.	.	.	1	.	1	1	1	.	.	.	.	.	.	.	.	.	.	.	.	.
Carex spicata	c	.	.	.	1	.	.	1	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Deschampsia flexuosa	c	.	2	.	2	2	3	.	2	3	.	2	2	.	1	.	.	1	.	2	2	2	2	4	.	.	2	1	.	1	.
Equisetum sylvaticum	c	.	.	.	.	.	.	1	.	1	.	1	2	2	.	1	1	.	.	.	.	.	.	.	.	.	1	1	2	.	2
Hypericum perforatum	c	.	.	.	1	.	.	1	1	.	.	.	1	.	.	.	.	.	.	1	.	.	1	1	.	.	.	1	1	.	1
Luzula pilosa	c	2	1	2	2	1	.	1	1	.	.	.	.	.	1	1	1	1	2	2	.	.	.	.	.	.	.	.	.	1	.
Maianthemum bifolium	c	3	1	2	.	1	.	.	.	.	.	1	.	.	.	.	2	1	1	.	2	.	.	.	.	.	2	.	.	.	.
Mycelis muralis	c	3	1	1	.	.	.	2	1	1	.	.	2	1	.	1	2	2	2	2	1	.	2	.	.	.	.	1	.	.	.
Oxalis acetosella	c	3	2	.	2	.	.	1	1	.	1	2	2	3	.	2	3	2	.	2	.	.	1	.	.	.	2	.	1	.	2
Polygonum persicaria	c	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	1	.	.	.	1	.	.	.
Pteridium aquilinum	c	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	3	4	.	4	4	.	3	4	.	4	4	.	.	3	.
Rubus saxatilis	c	2	.	1	.	1	1	2	.	1	.	1	1	.	.	.	3	.	.	1	.	.	3	.	.	1	.	.	.	.	.

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Rubus sp.	c	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	2	.	.	.	.	.	2	.	.	.	.	.	2	1	1
Tusilago farfara	c	1	.	3	1	.	.	1	.	3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Veronica chamaedrys	c	1	.	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	.
Carex pallenscens	c	.	1	1	.	1	.	1	1	.	.	1	.	.	.	.	.	.	.	.	1	.	1	.	.	.	.	.	.	.	.
Cruciata glabra	c	2	.	2	3	2	.	.	1	.	2	1	2	.	.	.	1	.	1	.	.	.	.	.	.	1	.	.	.	.	.

Occasional species: *Quercus rubra*(r), *Mentha arvensis*(1)

Appendix 2. *Detailed characteristics of collected soil samples (M – mounds, S – surrounding area)*

Plot	pH (H_2O)	EC (µS)	OM (%)	$CaCO_3$ (%)	Cd (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})	Fe (mg kg^{-1})
M1	5.21	73	13.07	0	1.15	56.65	184.08	59986.93
M2	4.59	79	13.11	0	0.77	85.42	157.86	69695.84
M3	5.08	72	13.10	0.215	1.03	48.95	149.80	54857.24
M4	5.57	60	11.45	0.084	0.43	28.54	136.33	76652.67
M5	4.63	162	26.13	0.00	2.52	116.47	152.34	35783.39
M6	4.9	181	28.51	0.123	4.08	187.13	265.38	44389.89
M7	5.12	59	11.17	0	0.41	39.71	125.79	46078.20
M8	4.75	60	14.16	0	1.31	44.76	148.92	35425.22
M9	4.6	67	16.47	0.213	1.33	76.83	136.82	54100.29
M10	5.82	207	25.76	0.294	2.97	128.25	250.57	46091.42
M11	5.46	96	14.00	0.211	0.19	25.77	114.34	35899.92
M12	4.67	96	12.16	0.343	0.90	36.88	151.24	33975.23
M13	4.37	102	18.68	0.583	0.47	32.76	124.41	31677.81
M14	4.42	103	13.65	0	0.39	37.54	141.12	41204.03
M15	4.93	174	18.49	0.164	2.62	82.56	268.98	115742.41
S1	4.42	76	11.13	0.083	0.47	63.21	61.76	11306.04

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S2	4.15	44	3.98	0.042	0.22	28.24	10.34	399.58
S3	4.03	68	5.07	0	0.21	21.59	13.61	3135.83
S4	4.32	137	34.62	0.206	2.04	165.01	75.91	13272.69
S5	3.5	168	17.44	0	0.41	83.83	45.85	3653.62
S6	3.62	81	12.11	0	0.53	66.25	35.06	2050.26
S7	4.23	32	3.74	0	0.03	12.55	4.99	392.44
S8	3.91	86	18.46	0.119	0.80	75.03	80.65	11508.58
S9	4.53	82	11.45	0.161	0.58	89.48	52.01	9032.99
S10	3.86	70	17.77	0.128	0.21	38.51	20.46	2052.99
S11	4.55	69	10.34	0	0.35	27.47	19.72	4593.73
S12	4.64	67	7.86	0.042	0.51	38.90	56.29	17562.58
S13	4.21	69	15.62	0.295	0.45	67.52	42.11	8078.88
S14	3.77	103	12.95	0.169	1.48	106.89	45.88	6314.13
S15	3.68	140	13.31	0	0.44	59.55	21.11	1295.97

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FACTORS INFLUENCING ABUNDANCE AND SPECIES RICHNESS OF OVERWINTERED WATERBIRDS IN PARISHAN INTERNATIONAL WETLAND IN IRAN

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Abstract. Parishan International Wetland, an Iranian Ramsar Site in Fars Province, is one of the two demonstration sites for the UNDP/GEF Conservation of Iranian Wetlands Project. The aim of this study was to examine the factors influencing water bird abundance, density, the number of species, and species richness in Parishan wetland from 1991 to 2010. We used remote sensing techniques to study some environmental factors affecting waterbirds community. Spearman's Correlation Coefficient and linear regression were also used to examine the impact of environmental factors on the community of waterbirds. The results showed that only the nearest wetland area was significantly correlated with abundance and density of species ($p < 0.01$). Also, the vegetation cover surface of wetland ($P < 0.01$); the deepest depth of wetland ($P < 0.01$); and the areas shallower than 1m ($P < 0.05$) were significantly correlated with the number and richness of species (Margalef Index). The Resulting models of the backward multiple regression test also indicated that the nearest wetland area was a good predictor of abundance (P value < 0.01) and density of waterbirds (P value < 0.05). Furthermore, the vegetation cover surface of wetland, was a good predictor of the number of species (P value<0.01) and of the richness species based on Margalef Index (P value<0.01). The area of the Parishan wetland has fallen very low in 2010 and reducing the area of the wetland has led to a sharp decrease in the number of birds in the wetland.
Keywords: *species richness, remote sensing, Fars province, wetland, bird abundance*

Introduction

Conservation of wetlands has become a frequent topic among wildlife managers (Kumar et al., 2007). Wetlands are important conservation sites due to their rich biodiversity; they are among the most productive ecosystems in the world and they harbor many globally threatened species (Casado and Montes, 1995; Green, 1996;

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Petrie, 1998; Getzner, 2002; Kumar et al., 2007). Wetlands have one of the highest biodiversity and biological productivity in the world (Whittaker and Likens, 1973; Gibbs, 1993; Casado and Montes, 1995; Paracuellos and Tellería, 2004). One of the most important functions of wetlands is to protect biodiversity. In fact, biodiversity envelops all forms of life on the planet and includes all genes, ecosystems, species, and ecological processes in the world (Balton et al., 2002; Collwell and Dodd, 1995; Behrouzi-Rad, 1996). Preserving the genetic diversity of species and ecosystems guarantees continuity of the environment. So, for the continuation of the health of the environment, identification of species, their habitats, and also the study of their population dynamics based on scientific methods are required (Mori et al., 2001; Mehrjoo, 1992). The importance of aquatic habitats for dependent organisms as irreplaceable ecosystems and preservation of biodiversity of valuable plant and animal species, has attracted the attention of protective agencies to these areas (Balton et al., 2002; Collwell and Dodd, 1995). Aquatic birds are considered as the most significant animal species to detect ecological changes in the aquatic environment and mangroves (Bambang, 2008; Bayly and Gomez, 2008). Waterbirds are important biological indicators that play an important role for the determination of the health of the wetlands (Amat and Green, 2010; Hoyer and Canfield, 1994; Sonal et al., 2010). Therefore, studying changes in the populations of Waterbirds and the factors involved in these changes is essential for the management of wetlands. Understanding factors that determine population size is central to ecology, population genetics, and conservation biology (Backwell et al., 1998; Frankham et al., 2002; Taft et al., 2002). Water bird community dynamics are complex and influenced by many natural and anthropogenic factors (Mundava et al., 2012). Natural dynamics of water bird populations is mainly affected by rain or by having access to water (Paillisson et al., 2002). Other factors affecting the community composition and abundance of birds include migration, breeding, and moulting of birds along with human factors (hunting, Water Harvesting and Agricultural activities) (Caziani et al., 2001; van Niekerk, 2010; Mundava et al., 2012). The aim of this study is to assess the changes in waterbirds community and the factors affecting these changes in Parishan wetland in Fars province in Iran.

Materials and methods

Study area

Lake Parishan, an Iranian Ramsar Site in Fars Province, is one of the two sites nominated for the UNDP/GEF Conservation of Iranian Wetlands Project. As a part of Arjan Parishan Protected Area, Parishan Lake was registered by UNESCO as a Biosphere Reserve (Department of Environment of Fars, 2010). This wetland is located in the eastern part of Kazeroun City surrounded by Parishan protected area (29° 34' 48" N and 51° 54' 36" E) with an area of about 60000 hectares in southwest Iran (*Fig. 1*). Parishan wetland with an arid and desert cold climate at an average elevation of 820 m above sea level receives an annual rainfall of about 430 mm (Department of Environment of Fars, 2010). The evaporation capacity in the area is high (2470 mm/yr on average) ranging between 1600-3350 mm/yr. The surface area of the water body changes

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seasonally according to the hydrological condition and generally varies from more than 2500 to almost 5000 ha. The Lake does not have a natural outflow and its main source of water loss is through evaporation and consumption by vegetation cover. However, a large number of deep wells (more than 800) have been dug around the Lake exploiting significant volume of groundwater for agricultural uses resulting in wetland discharge (Fars DOE, 2010). The wetland is almost surrounded by agricultural farms in all directions; however, further on the northern elevations, there exists a semi-dry type of forest cover consisting mainly of scattered oak trees. The water body of the Lake as well as different patterns of vegetation cover around and inside the lake provides diverse habitats which supports the rich biodiversity of the wetland. The Lake hosts significant number of migratory waterbirds specially wintering population which breed there. At least, five globally threatened species such *as Pelecanus crispus, Marmaronetta angustirostris, Aythya nyroca, Oxyura leucocephala* and *Aquila heliaca* (Fars DOE, 2010) are usually present on the lake, occasionally in large population.

Figure 1. *Study area: Geographical location of Parishan Wetland in Fars Province, Iran (Fars DOE, 2010)*

Waterbird surveys

The avian characteristics monitored for each year are presented in *Table 1*. The Environmental Protection Agency of Iran is doing the mid-winter count of waterbirds in Parishan wetland every year. Information about the counting of waterbirds over the past years was obtained from the Environmental Protection Agency of Iran and Waterbird surveys were conducted from 1990 to 2015. To calculate the species richness the index of Margalef was used (*Table 1*).

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Table 1. *The investigated characteristics of birds*

Avian characteristics	Abbreviation	Equation
The number of species in each year	S	
The density	D	average value of the number of individuals per ha
Total number of all birds in the wetland in each year	N	
Margalef species Richness	R	$R = \frac{s-1}{\ln N}$

S = the number of Species, N= Total size of population, Pi= Relative abundance of species i, ni= Number of species i

Environmental characteristics

All environmental factors monitored are shown in *Table 2*. Landsat satellite images were used to calculate the area of the wetland, shoreline length, the deepest depth of the wetland, coverage of water surface by vegetation and the area of the nearest wetland (Arjan wetland) in each year. Firstly, radiometric and geometric corrections were applied to all images using ENVI 5.1 Software. Then, the layers of the coverage of water surface by vegetation and water area were prepared using supervised classification method.

Table 2. *Environmental and human factor descriptions and abbreviations*

Factors	Abbreviation	Measuring unit
Wetland area	Wa	hectare
Open water area	OWa	hectare
Vegetation cover	Vc	hectare
Shoreline length	Sl	m
Area shalower than 1 meter	As	hectare
The nearest wetland area	Nw	hectare
Average temperatures	At	C0.
Shoreline Development Index	SDI	
Most depth	Md	m

To calculate the water areas with a depth of less than 1 m and the deepest depth of the wetland in each year, the bathymetric maps of the wetland in different years were obtained from the Environmental Protection Agency of Fars province and on the basis of bathymetry maps of the wetland, the DEM layers of the wetland area was prepared by the ARC gis 10.2 Software. Then, the DEM layers of the wetland were cropped for each year according to the water surface area of the wetland. Finally, these layers were entered into the ARC gis Software and the water areas with a depth of less than 1 m and the deepest depth of the wetland were calculated for each year.

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Temperature data were obtained from the weather station of the Parishan wetland and average temperatures for the winter months (when counting birds) were calculated.

Shoreline development was calculated based on the following equation for each year (Margalef, 1983):

$$SDI = \frac{Sl}{2\sqrt{OWa \times \pi}} \quad \text{(Eq. 1)}$$

where SDI stands for Shoreline Development Index; Sl stand for Shoreline Length; and OWa stands for Open Water Area

Data analysis

Abundance data were first transformed into densities (number of birds per ha) to allow comparison of the wetland with different sizes in different years. Square-rooted densities were numerically transformed to down weight dominant species that could have given erratic counts over the replicated samples (Niu et al., 2013).

The Shapiro-Wilk test was used to test for normality on all variables analysed (SPSS 18.0); and if non-normal variables transformed logarithmically or trigonometrically (Jobson, 1992; Atmar and Patterson, 1993; Sokal and Rohlf, 1994). But transformations did not stabilize variance of some independent variables.

Therefore the Spearman's Correlation Coefficient (r) was used for simple relation analyses with the variables. Then, a backward multiple regression test was employed for modeling the relation between the number of species, the species density, total number of birds, and Margalef species Richness Index as the dependent variables and the characteristics of the wetland as the independent variables.

In this method, all variables are first entered into the model and then, the least important variable is removed according to removal criteria; this process continues until all the less important variables are gradually removed. Finally, the final model will be calculated based on the main variables. Therefore, all the remaining variables have an acceptable and significant correlation with each other. Models obtained by backward method are superior than those made by Enter and Stepwise methods in terms of the number of variables. That is to say, the number of variables is not as many as that in the Enter models and not as few as that in the Stepwise models. Also in these models, higher correlation between the calculated performance and actual performance is observed compared to the Stepwise model. Logistic regression is used for modeling the relation between binary dependent variable and one or more environmental predictor variables. In other words, logistic regression can be used to predict the dependent variable based on the predictor variables. Formula of Backward Model is given in *Equation 2*.

$$Yi = \beta 0i + \beta 1iX1i + \beta 2iX2i + \beta 3iX3i + \beta 4iX4i + \ldots + \beta(p-1)i\, X\,(p-1)i \quad \text{(Eq. 2)}$$

Yi= the linear predictor

β0i= Constant coefficient

β1i - β4i= the coefficients of the variables

X1i - X4i= Variable values

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Results

Table 3 lists the dependent variables (characteristics of birds) measured and *Table 4* lists the independent variables (characteristics of Wetland) measured in different years and *Figs. 2* and *3* show the maps of vegetation cover and water surface of the wetland in different years.

Table 3. *Characteristics of birds in different years*

Year	N	D	S	R
1991	27126	6.93	39	3.72
1992	25500	6.51	54	5.22
1993	10171	2.32	38	4.01
1995	21275	5.17	57	5.61
1999	6076	1.36	26	2.86
2000	11142	2.48	36	3.75
2001	8591	2.02	47	5.07
2002	4172	1.03	25	2.87
2003	20970	6.11	35	3.41
2005	5000	1.22	32	3.63
2007	24561	7.01	37	3.56
2009	253	0.84	11	1.80

Table 4. *Characteristics of the wetland in different years*

Year	Wa(ha)	OWa(ha)	Vc(ha)	Sl(m)	As(ha)	Nw(ha)	At(C0)	SDI
1991	3909.06	3070.8	838.26	85740	491.04	1141.94	11.6	4.36
1992	3914.91	3021.12	893.79	88740	880.49	1245.51	9.9	4.55
1993	4383.09	3570.3	812.79	101340	876.95	1782.87	12.3	4.78
1995	4107.42	3166.47	940.95	98340	882.97	1444.76	13.9	4.93
1999	4444.38	3635.58	808.8	110700	858.58	1921.17	10.6	5.18
2000	4491.0	3526.83	964.17	112900	866.32	1729.99	12.9	5.36
2001	4249.26	3314.44	934.82	110250	778.8	1850.26	12.7	5.40
2002	4026.87	3232.48	794.39	97570	526.9	2012.03	12.3	4.84
2003	3428.1	2861.18	566.92	79360	507.89	1498.60	12.4	4.18
2005	4091.85	3220.14	871.71	97980	450.3	1980.79	13.7	4.87
2007	3503.79	2680.19	823.6	82460	423.85	1295.92	13.0	4.49
2009	299.16	111.69	187.47	14100	111.69	1300.46	11.5	3.76

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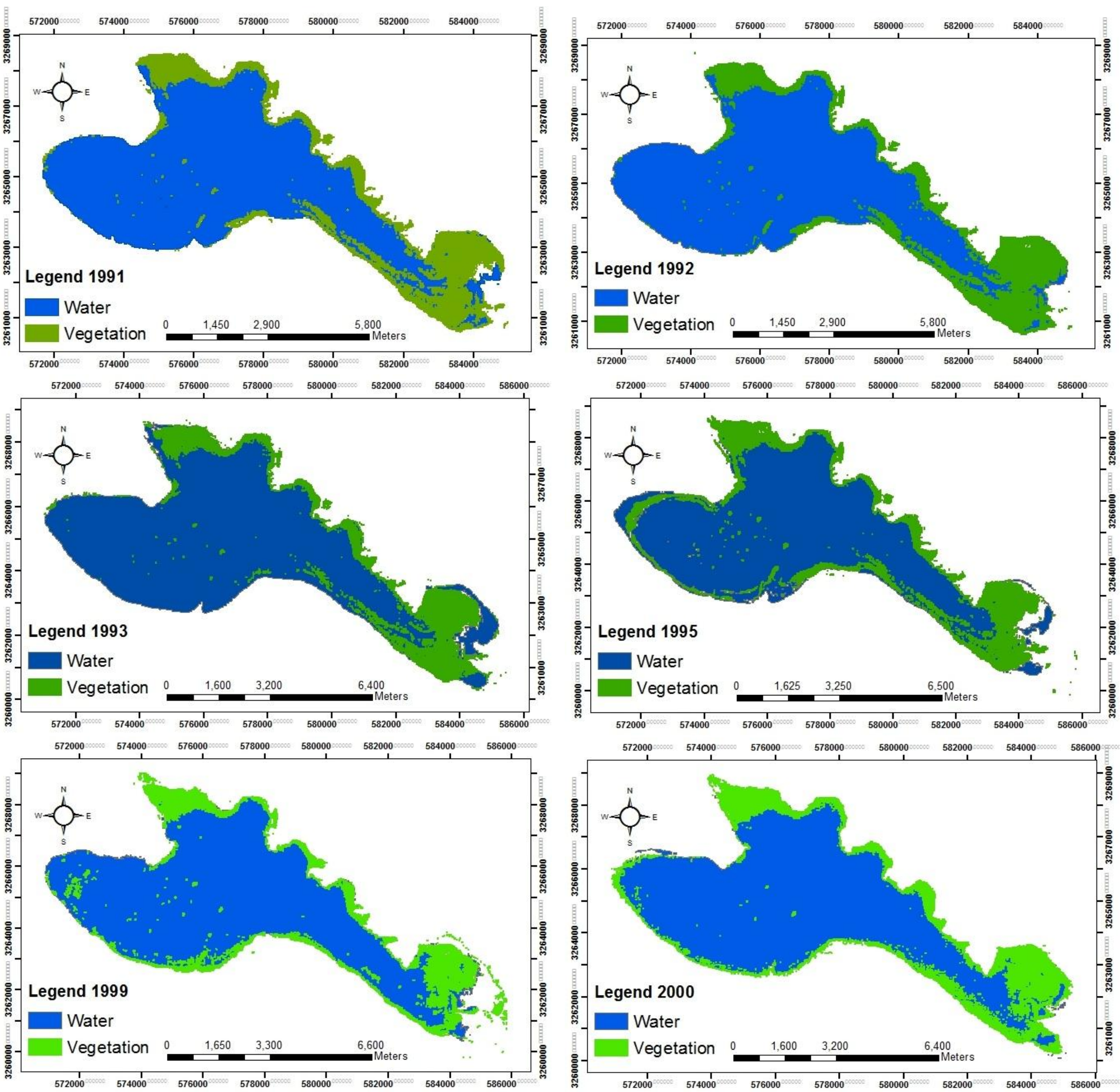

Figure 2. *Classes of water and vegetation of Parishan wetland during the different years*

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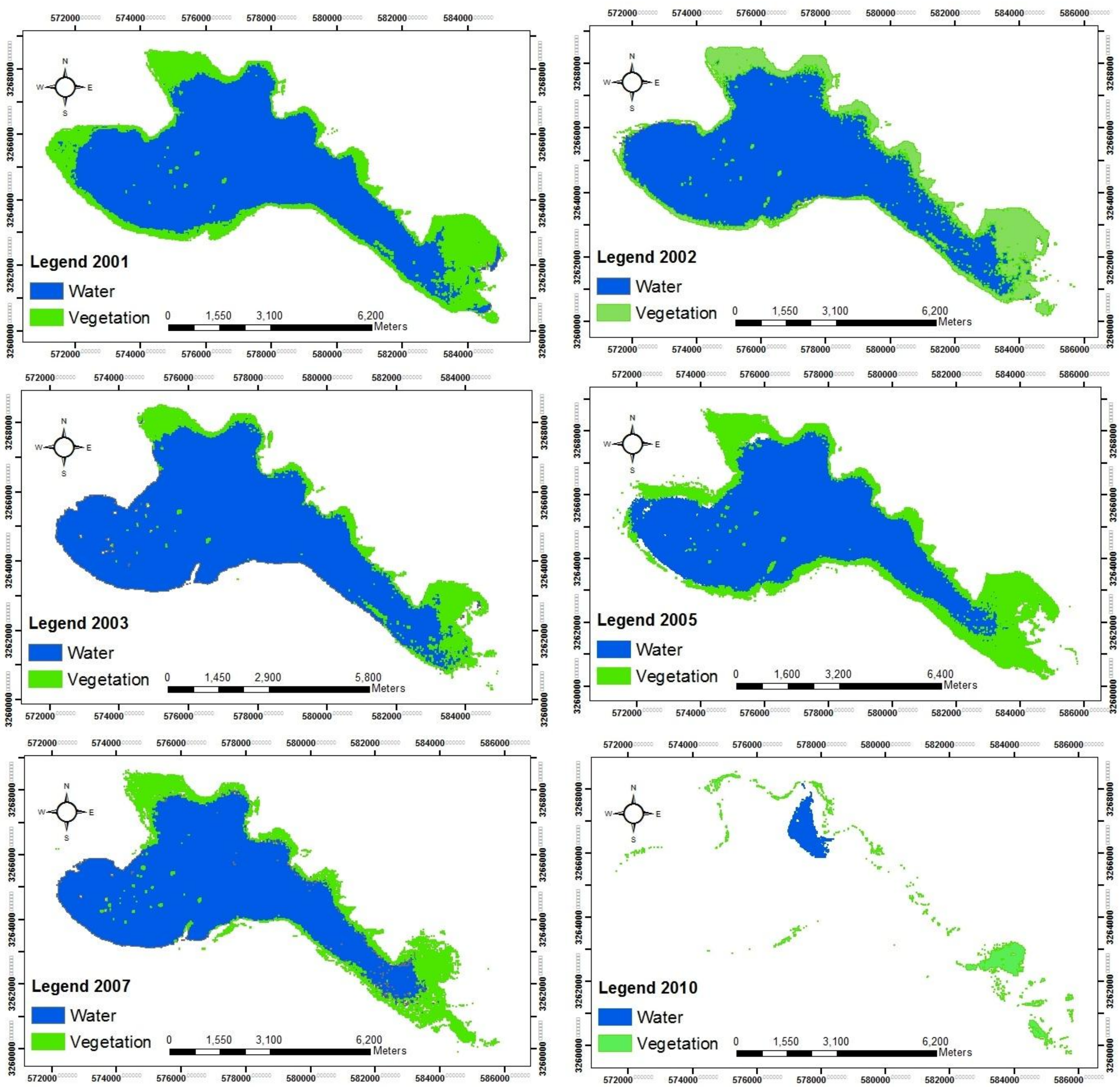

Figure 3. *Classes of water and vegetation of Parishan wetland during the different years*

Only one environmental variable was significantly correlated with the total members of species and the density (the number of waterbirds per ha) ($P < 0.01$) (*Table 5*). The correlation between these variables was negative. They were entered into backward multiple regression tests for modeling to predict the frequency and density of the waterbirds (*Table 6* and *7*).

Also, three environmental variables (vegetation cover surface of the wetland ($P < 0.01$); the deepest depth of the wetland ($P < 0.01$); and the areas shallower than 1m ($P < 0.05$)) were significantly correlated with the number of species (*Table 5*). They were entered into backward multiple regression tests for modeling to predict the number of species (*Table 8*).

Also, three environmental variables (vegetation cover surface of the wetland (P < 0.01); the deepest depth of the wetland (P < 0.01); and the areas shallower than 1m (P < 0.05)) were significantly correlated with the Margalef species Richness index. (*Table 5*). They were entered into backward multiple regression tests for modeling to predict the Margalef species Richness index (*Table 10*).

Table 5. *Statistical relationships (r) between the environmental characteristics and the characteristics of water bird species in different years in Parishan wetland. Level of significance: * P < 0.05, ** P < 0.01.*

Variables	N		S		D		R	
	r	P	r	P	r	P	r	P
Wa	0.133	0.681	0.189	0.557	0.196	0.542	0.378	0.226
OWa	-0.266	0.404	0.014	0.966	-0.322	0.308	0.182	0.572
Vc	0.434	0.159	0.713**	0.009	0.343	0.276	0.825**	0.001
Sl	-0.140	0.665	0.203	0.527	-0.203	0.527	0.385	0.217
As	0.294	0.354	0.601*	0.039	0.168	0.602	0.706*	0.010
Nw	-0.748**	0.005	-0.469	0.124	-0.720**	0.008	-0.245	0.443
At	0.049	0.880	0.217	0.498	0.119	0.712	0.315	0.318
SDI	-0.189	.557	0.224	0.484	-0.238	0.457	0.399	0.199
Md	0.371	0.235	0.760**	0.004	0.249	0.436	0.816**	0.001

Table 6. *The models obtained by the backward multiple regression test using the number of water bird species as dependent variables and the area of the nearest wetland as the independent variables*

Parameters included in the model	r2	F	Coefficient	P
Model	0.509	10.381		
Constant			48412.324	0.001
Nw			-21.668	0.009

Table 7. *The models obtained by the backward multiple regression test using the density of water bird species per hectare as the dependent variables and the area of nearest wetland as the independent variables*

Parameters included in the model	r2	F	Coefficient	P
Model	0.466	8.713		
Constant			3.808	0.003
Nw			-0.002	0.014

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Table 8. The models obtained by the backward multiple regression test using the number of species as the dependent variables and the area of vegetation cover of the wetland areas shallower than 1 meter and the deepest depth of the wetland as the independent variables

Parameters included in the model	r2	F	Coefficient	P	Excluded Variables	P
Model	0.521	10.868				
Constant			3.019	.779		
Vc			0.042	.008		
					As	0.223
					Md	0.441

Table 9. The models obtained by the backward multiple regression test using the Margalef species richness index as the dependent variables and the area of vegetation cover surface of the wetland areas shallower than 1 meter and the deepest depth of the wetland as the independent variables

Parameters included in the model	r2	F	Coefficient	P	Excluded Variables	P
Model	0.517	10.705				
Constant			0.946	0.318		
Vc			0.004	0.008		
					As	0.305
					Md	0.830

Discussion

Our results showed the importance of the nearest wetland area in explaining the frequency and density of waterfowl in Parishan wetland. Our results showed that when the nearest wetland area attracted an abundant number of birds, the Parishan wetland area decreased. It means there is no rise in competition, i.e. the birds occupy both wetlands to prevent increased competition for food, shelter, nesting, etc. But, when a wetland area decreased they migrated to nearby wetlands to satisfy their biological needs. Many studies were conducted on the effect of isolation (distance to the nearest wetland) as a variable affecting the abundance of waterbirds (Brown and Dinsmore, 1986; Craig and Beal, 1992; Andrén, 1994; Rosenberg et al., 1997; Tellería and Santos, 2001), but we did not find any report on the impact of the nearest wetland area on the frequency and density of waterfowl.

At the beginning of our study, we assumed that the area of wetland had a significant impact on the abundance of birds, but the results showed that wetland size had no significant effect on the bird abundance. The results of this study performed on the effect of wetland area on the abundance of birds were consistent with those obtained by Sulaiman et al. in 2015. But, other studies showed a significant relation between an increase in the number of birds and that in the size of wetlands (He and Legendre, 1996).

The models obtained by the backward multiple regression test indicated that the nearest wetland area was a good predictor of frequency (P value < 0.01) and density of waterbirds (P value < 0.05).

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The results also showed that three environmental factors (area of vegetation cover of the wetland; the deepest depth of the wetland; and the areas with depths less than one meter) had significant relation with the number of species. Number of bird species may increase as a result of an increase in habitat heterogeneity (He and Legendre, 1996; Elmberg et al., 1994; Sulaiman et al., 2015). A more heterogeneous range of habitats allows the co-occurrence of more species because they meet the habitat requirements of more species (Sulaiman et al., 2015). Also, more species may occur in areas of more diverse habitat because of spatial segregation that reduces competition (Sulaiman et al., 2015) and these three factors can increase heterogeneity of the wetlands. Other studies conducted in wetland ecosystems have demonstrated the importance of habitat heterogeneity (Svingen and Anderson 1998; Fairbairn and Dinsmore 2001; Riffel et al. 2001; Gonzalea-Gajardo et al., 2009).

Furthermore, the results of this study showed a significant relation between the Margalef species richness index and three environmental factors (area of vegetation cover of the wetland; the deepest depth of the wetland; and the areas with depths less than one meter). Increasing the richness species index as well as increasing the number of species may increase as a result of an increase in habitat heterogeneity; these three factors can increase heterogeneity of the wetlands.

Other studies have shown a significant relation between the size of habitats, the number of species, and richness species (Sillén and Solbreck 1977; Brown and Dinsmore 1986; Opdam 1991; Andrén 1994; Turner 1996; Tellería and Santos 2001), but this study found that the wetland size had no significant effect on the number of species and richness species index.

The models obtained by the backward multiple regression test indicated that among these three variables, the vegetation cover surface of the wetlandwas a good predictor of the number of species (P value<0.01) and the richness species index of Margalef (P value<0.01).

Conclusion

The result showed that abundance was fundamentally affected by the nearest wetland area which required integrated management of adjacent wetlands.

Also, the results showed that the number of species had a significant relation with the vegetation cover surface of the wetland, the deepest depth of the wetland, and the areas shallower than 1m. These 3 factors play a major role in habitat heterogeneity. So, to increase biodiversity, habitat heterogeneity should be managed at an appropriate level.

Wetlands provide ecological functions such as protective nursery habitat for fish and shellfish, erosion prevention, flood protection, and water filtration (Dahl, 2005; Behrouzi-Rad, 2014). They also provide vital feeding, resting, and breeding habitat for resident and migrating birds. Seabirds and colonial waterbirds face threats to their habitats and sites on which they depend (Behrouzi-Rad, 2014); conservation of seabirds and colonial waterbirds is a local matter. Nesting and roosting seabirds and colonial waterbirds are particularly affected by local conditions (Behrouzi-Rad, 2014). Parishan wetland is one of the two demonstration sites for the UNDP/GEF Conservation of Iranian Wetlands Project. The water body of the Lake as well as different patterns of vegetation cover around and inside the lake provides diverse habitats which supports the rich biodiversity of the wetland. The

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Lake hosts significant number of migratory waterbirds which use it for wintering, feeding, breeding, and stationing. The higher records of waterbirds population in the Lake exceed 120,000 (1970s and 1980s). (DOI, 2010). In 7 out of 17 years of accessible records since 1990, the annual counts of waterbirds in Lake Parishan have exceeded the 20,000 Ramsar threshold for internationally important wetlands. Also, *Podiceps cristatus*, Great Crested Grebe, *Phalacrocorax pygmaeus* Pygmy Cormorant, *Anser anser*, Greylag Goose, *Oxyura leucocephala* White headed Duck, *Larus ridibundus* Black Headed Gull, and *Tadorna ferruginea* Ruddy Shelduck have been recorded in numbers exceeding %1 of their bio-geographical population. At least, five threatened species are usually present in the lake and occasionally in noticeable population. These are *Pelecanus crispus*, *Marmaronetta angustirostris, Aythya nyroca, Oxyura leucocephala*, and *Aquila heliaca* (DOI, 2010). Unfortunately, the Parishan wetland has completely dried up since 2011.

The wetland is almost surrounded by agricultural farms in all directions. Water for irrigation is supplied from groundwater wells. Now, there are more than 940 water wells around the wetland and the water extracted from these wells is used in agriculture (DOI, 2015). Excess exploitation of water wells has brought about a drop in underground water level by 15.11 meters between 1990 and 2015 (WAOI, 2015). As long as the resources of underground water are not restored, there is no chance of restoring the wetland because all the water entering the wetland, penetrates into the ground.

Agricultural sector imposes agreat pressure on the wetland due to excessive withdrawals of water from the wells, digging water wells, and excess use of fertilizers and pesticides to increase productivity. Therefore, until rigorous and scientific management is not practiced in agricultural land around the wetland, it is not possible to manage the wetland and protect its ecological benefits.

Therefore, drying up of springs, reduction of the level of underground water, an increase in organic and inorganic contaminants, and finally drying up of the wetland are all resulted from uncertain effects of land use changes along with climate changes.

As shown in *Figure 3*, the area of the Parishan wetland has fallen very low in 2010 and reducing the area of the wetland has led to a sharp decrease in the number of birds in the wetland.

Parishan wetland has completely dried up from 2011 onwards. Along with the increased discharge of water from wells surrounding wetlands, the underground water level and the volume wetland water also have declined and the wetland has completely dried up in 2011. Most of wetland water is supplied through precipitation and spring water and a portion is supplied through groundwater flow; this is while the amount of annual precipitation in the region is very small compared to the rate of evaporation from free surface of the wetland. The average evaporation in the study area during the studied period has been equal to 2731.8 mm; and considering the coefficient of evaporation pan (0.7) (Zamin Ara Consulting Engineers of Fars, 2011), evaporation from the wetland surface has been equal to 1912.26 mm. Now, given that the average area of wetland during the studied period was 24 square kilometers, it follows that the average annual evaporation from the wetland surface has been equal to 38.245 million cubic meters, while the average rainfall has been equal to 8.892 million cubic meters. On the other hand, the water of springs around the wetlands is consumed by farmlands before reaching the wetland; and in recent years, most springs have dried up due to drought and low levels of underground water. Meanwhile,

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digging a large number of wells around the wetlands and depletion of groundwater have caused the groundwater level go down 13.68 meters; and now, there is no possibility of providing water of wetland by underground water flows (Jahanbakhsh Ganjeh et al, 2017).

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WATER USE EFFICIENCY OF RICE AND SOYBEAN UNDER DRIP IRRIGATION WITH MULCH IN THE SOUTH-EAST OF KAZAKHSTAN

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Abstract. Water saving was and remains an acute problem in places with water scarcity and inaccessibility. In order to solve this problem, drip irrigation started to be used in many countries, and for the first time, in Kazakhstan, there were used cultures that consume water sufficiently in large quantities such as rice and soybean. Ways of irrigation have significant effect on elements of structure of a rice and soybean crop. Drip irrigation with a two-row tape way of crops forms essentially distinct structure of a rice crop in comparison with usual technology of cultivation of rice and soybean. Rice (*Oryza sativa* L.) and soybean (*Glycine Max* L. Merr.) crops were grown in 2013-2016 to determine effect of drip irrigation in the different variants water levels on growth and productivity under conditions of the south-east of Kazakhstan. In result of three years of researches on studying of rice and soybean drip irrigation efficiency were made the following conclusions: Drip irrigation (Di) with application of the mulching film promotes emergence of early and amicable shoots of rice and soybean, intensive growth and development of plants, productivity increase. Drip irrigation with use of the mulching film at 8-10 times reduces a contamination of crops of rice that allows to exclude application of manual use, mechanical and chemical processing of crops. Use of the mulching film under rice drip irrigation promotes creation of the favorable nitric mode of the soil with big accumulation of ammoniac and nitrate nitrogen and mobilization of motile phosphorus. The best way of rice crops under drip irrigation is 2-row tape crops with distance between tapes of 70 cm and a row-spacing in a tape of 30 cm. At the same time the drip tape keeps within row-spacings in a tape. Comparison of the productivity of soybean varieties showed that productivity in variants with mulch far exceeded variants without mulching.
Keywords*: Oryza sativa L., Glycine Max L. Merr., water-saving technologies, productivity*

Introduction

Irrigated agriculture, the major contributor of agricultural production, faces the challenge of improving irrigation water use efficiency and meanwhile ensuring food security (Li et al., 2016a). The global water consumption for irrigation has been steadily growing over the last 50 years and today it makes 70% of all water consumption (Tian et al., 2016). The great challenge of the agricultural sector is to produce more food from less water, which can be achieved by increasing Crop Water Productivity (CWP) (Zwart, 2004). Deficiency of fresh water increases in high places around the world.

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According to forecasts of FAO and IFPRI global demand for water resources according to the scenario of usual development by 2030 will increase twice. The Water Resources Committee of the Ministry of Agriculture deals with the management and allocation of water resources and water use. This situation often makes it difficult to conduct complex, integrated scientific research. State and private land ownership is recognised and equally protected. However, it is difficult to deal with the maintenance of the irrigation systems for small peasant farmers. Government support is required. Public-private partnership should be considered to introduce the most advanced water-saving technologies (FAO).

Especially critical situation with water supply is predicted in the countries of Central Asia because of the growing intake of water, thawing of glaciers and droughts, the processes of degradation of lands and desertification. For Kazakhstan with strong dependence on climate change and cross-border water currents, the scenario of usual development in principle does not allow to solve problems of water supply of economy and the population in the near future.

The main consumer of water resources in Kazakhstan, as well as in other countries of the Central Asian region, is the irrigation whose share is over 70% of all potential of a superficial drain. Practically in all territory of the Republic the intense water management situation is caused by a lack of water resources and pollution of water sources is present (Paramonov, 2009; Kwan et al., 2011). Annual deficiency of water in Kazakhstan makes 2-3 km^3. The problem of water dependence can be dangerous to national security of Kazakhstan because of emergence of the interstate and regional conflicts (the cross-border rivers) (Water resources of Kazakhstan in the New Millennium: 2004). Alternative irrigation layouts and water management approaches could contribute to reduced water use and increased irrigation efficiency (Beecher et al., 2006).

The greatest consumer of irrigation water per unit area is rice. Rice (paddy) is the second most important commodity worldwide, and rice cropping fields significantly contribute to climate change since they are a considerable source of methane (Coltro et. al., 2016). Rice paddy agricultural methods require a large quantity of water (Kang et al., 2007). Statistics indicate that the water consumption of rice accounts for approximately 54% of the total water consumption (He et al., 2014); and more than 50% of reserves of irrigation water is spent on cultivation of this culture in Kazakhstan. In Kazakhstan, as well as in the countries of Central Asia, the way of cultivation of rice based on continuous flooding of crops is accepted. Under production conditions the irrigating norm of rice with continuous flooding and pro-accuracy changes ranging from 25 to 35 thousand m^3/hectare. Under this method of an irrigation a consumption of irrigation water on rice cultivation greatly exceeds biological need of plants for water which considerable part is lost on evaporation, filtration and feeding of ground waters. That is why rice irrigation by method of flooding is one of methods of melioration of highly salinized lands and way of fight against weeds weed control. Cultivation of rice with an expense of a huge amount of irrigation water within more than 50 years has brought to an environmental problem of Aral and in recent years of Balkhash, too. Long-term cultivation of rice with flooding of checks has led to sharp decrease in efficiency of use of the irrigated lands with considerable reduction of the areas and efficiency of other irrigated cultures. Now rice in Kazakhstan is cultivated on the area of about 100 thousand hectares. For cultivation of rice on such square about 3 billion m^3 of irrigation water are annually spent.

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In this regard, undoubted relevance is acquired by the researches directed to the development of systems of agriculture and technologies providing effective use of natural and water resources, increase of efficiency of the irrigated arable land and have a strategic importance, as in national, and international scale. There is a need for an agriculture intensification, use of new breakthrough high technologies for crop cultivation. The developed nature protection technology of cultivation of rice is suitable for distribution on the irrigated lands of a zone of paddy culture in Uzbekistan, Tadzhikstan, Turkmenistan, introduction of which will provide improvements of an ecological situation in the region of the Aral-Syrdaryinsky basin and in general Central Asia.

Many water-saving technologies are currently used in rice production, including alternate wetting and drying irrigation, the rice intensification system, aerobic rice and the ground cover rice production systems (GCRPSs) (He et al., 2016). But the numerous researches, conducted mainly abroad, show that the most effective way of rational use of irrigation water is, without any doubts, the drip irrigation. Drip-irrigation is the slow and frequent application of small amounts of water (Zhu et al., 2013). Drip irrigation is the most effective way to supply water and nutrients to the plant and not only saves water but also increases yield of fruits and vegetable crops (Tiwari et. al., 2003), it also stabilizes the daily range of soil temperatures (Xing et al., 2012), which can benefit crop growth (Li et al., 2016b). Drip irrigation is a way of watering at which water is pumped in the small portions evenly to plant roots throughout all vegetative period and irrigational moisture goes only to plants, but is not spent for row-spacings. Because of it, many researchers have reported higher application efficiency of drip irrigation system over the conventional irrigation methods (Tiwari et al., 1998). Drip irrigation also has been used extensively to reclaim the salt-affected soils for crop productivity in recent years (Zhang et al., 2014). Efficiency of use of the mulching films under drip irrigation in preservation of moisture and control of weeds is proved (Raina, 1998; Seyfi, 2007). With the invention of new technologies and the equipment for drip irrigation, in foreign literature there is mentioned this method's efficiency in rice cultivation. In the separate experiments made in the USA, India, Australia, nowadays it is reported about efficiency of a combination of drip irrigation ways with zero processing of the soil or with use of the mulching films (Enciso-Medina et. al., 2001).

The use of plastic mulch in agriculture has increased dramatically in the last 10 years throughout the world (Kasirajan, 2012). Introduction mulched drip irrigation (MDI) has been widely employed in arid and semiarid regions for crop cultivation to accurately supply water and fertilizer in the soil in conjunction with soil evaporation reduction (Zhou et al., 2017). Mulched drip irrigation is a combination of the drip irrigation and the mulch technique (Liu et al., 2012). When soil temperature is insufficient, plastic mulch enhances tuber yield and water use efficiency (WUE) (Wang et al., 2011). Conventionally, mulches increase the yield and water use efficiency (WUE) to a great extent by augmenting the water status in the root zone profile (Mukherjee et al., 2010). Different types of materials like wheat straw, rice straw, plastic film, grass, wood, sand etc. are used as mulches. They moderate soil temperature and increase water infiltration during intensive rain (Yaghi et al., 2013). Drip irrigation with plastic film mulch (DI-PFM) can maintain high rice yields with significant water savings. Both plastic mulch and drip irrigation profoundly influence soil environment, especially through spatiotemporal changes in soil water (Zhang et al., 2017). However, rice seedlings often develop chlorosis when grown

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with DI-PFM on calcareous soil. Chlorosis in drip-irrigated seedlings occurs not because Fe availability is low but because the Fe uptake capacity of roots is weak at low temperatures (Zhang et. al., 2016). Rice plants are more sensitive to the HCO_3^- concentration of irrigation water under FI than under DI-PFM (Zhang et al., 2015). Water application systems under wells extracting groundwater are one of the major factors influencing climate change in the agricultural sector. In the context of growing demand for adaption of pressurized irrigation with electric pumps in south central India, the present study was undertaken to assess the carbon dioxide emission (CO_2) for different irrigation systems. It was observed that among all the irrigation systems, the drip system gave the lowest CO_2 indicating the maximum climate change mitigation potential in the irrigation sector of selected region under wells (Reddy et al., 2015). While straw mulch usually reduces soil evaporation and stabilizes soil temperature, hence increasing yield, this effect may depend on the irrigation water input conditions. Two experiments in lowland rice paddies in lao pm tested the effect of rice straw mulch under various water input-standard farmer condition to reduced input condition by using either drip irrigation or lower furrow irrigation water input by increasing the furrow irrigation interval before flowering- on growth and yield of sweet corn. The time course of water balance components was determined to elucidate the mechanisms of mulch and water input interaction for row planted maize after rice harvesting (Vial et al., 2015). The method involves performing seed preparation, soil preparation by preventing and treating a weed, seeding, seedling management, irrigation management, fertilizer management and pest control, where seedling density is 33.3 to 36 thousand holes per mu in seeding or the seedling density is 30.5 to 32.6 thousand holes per mu in seeding. Target yield and fertilization weight are determined. Nonflooded irrigation is an important water-saving rice cultivation technology, but little is known on its photosynthetic mechanism. The aims of this work were to investigate photosynthetic characteristics of rice during grain filling stage under three nonflooded irrigation treatments: furrow irrigation with plastic mulching, furrow irrigation with nonmulching, and drip irrigation with plastic mulching. Compared with the conventional flooding treatment, those grown in the nonflooded irrigation treatments showed lower net photosynthetic rate, lower maximum quantum yield, and lower effective quantum yield of psii photochemistry (He et al., 2014).

The grain yield ranged from 3.35x10(3) kg ha(-1) to 6.86x10(3) kg ha(-1) under plastic mulching drip irrigation, which was 19.3-60.31% lower than that under flooding irrigation. Correlation analysis showed that the roots at the a1 and b1 sites were positively significantly correlated with yield components and aboveground agronomic traits. Therefore, improving root development at the a1 and b1 sites at flowering stage could be a key factor to obtain higher grain yield and good agronomic performance under plastic mulching drip irrigation (He et al., 2013). The experiment demonstrates that the di treatment has greater water saving capacity and lower yield and economic benefit gaps than the fim and fin treatments compared with the cf treatment, and would therefore be a better water-saving technology in areas of water scarcity (He et al., 2015). Under non-flooded irrigation, root length was significantly reduced with more roots distributed in deep soil layers compared with the conventional flooding treatment; the drip irrigation treatment had more roots in the topsoil layer than the fim and fin treatments (Chen et al., 2015). The weeding method involves spraying the fencers and harrowing with soil sealing agent before seedling in

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rice drip irrigation planting. The rice is planted by process of the drip irrigation under the film after spraying the fencers with soil sealing agent for 5-7 days. The fencers are sprayed with herbicide after seeding in rice drip irrigation planting under a film when a rice seedling has 3-4 leaves.

In order to enhance the productivity of aerobic rice from present yield level, a production technology which has proved beneficial in increasing the productivities of both grain and water. Disclosed is combined effect of scheduling irrigation at 125% pan evaporation, application of 100% recommended doses of nitrogen, phosphorus and potassium (npk) fertilizers, azophosmet and humic acid through drip system. It is proved that drip fertigation technology is beneficial in boosting grain yield coupled with the increased values for water productivity besides improving the fertilizer use efficiency (Arulmozhiselvan, 2009). Drip irrigation is the future of irrigated agriculture, which needs to be strengthened by optimizing the technology for different crops (Sharda et al., 2016).

(Irmak, S. et al.,2014) evaluated the relative evaporative losses and water balance components in two soybean (*Glycine max* (L.) Merr.) fields under subsurface drip irrigation and center pivot irrigation systems in south-central Nebraska. Evaporation losses were estimated as the difference between measured evapotranspiration and estimated transpiration . Average soil water content in the crop root zone and effective rainfall were estimated using the water balance method.

Soybean (Glycine max) is one of the most important legume crops which fix atmospheric nitrogen in symbiotic association with bacteria through nodules. A study was conducted to investigate the effect of molybdenum and preinoculation of Rhizobium on biological nitrogen fixation and yield of soybean under drip irrigation system (Kanaan et al., 2013).

The growth and yield potential of soybean and the effects of mulching on desert sand were evaluated in relation to N accumulation in nodules. It was concluded that nitrogen fertilization improves the nutraceutical properties of soybean, although its effect depends on the activity of the isoflavone biosynthetic pathway and the concurrent extent of seed bulging (Vamerali et al., 2012).

A field experiment conducted during 2009 and 2010 at the research farm of the Indian Agricultural Research Institute, New Delhi to study the performance of soybean as influenced by the intercropping of cereals, viz. maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench.) and pearl millet (*Pennisetum glaucum* L.) with 0, 50, 75 and 100% of their respective recommended dose of N (RDN) levels. The highest gross returns, net returns, B: C ratio and soybean equivalent yield (SEY) were observed under soybean+maize intercropping system along with 100% RDN to intercropped maize in both the years of experimentation (Layek et al., 2015). Soybean grown under soybean-wheat-mungbean system had significantly higher total uptake N and K over soybean-wheat-fallow system (Prajapa et al., 2015). Impact of short-term zero tillage is short lived for favourable soil health and root parameters when skipping with conventional tillage (Rajkumar et l., 2015).

The water requirement of soybean and wheat estimated by Penmann-Monteith method was in close agreement (-2.58% and 9.26% deviation) with the measured average water requirement (401.6 and 352.2 mm) respectively followed by Hargreaves method for Bhopal district (Singh et al., 2015). Sharma et al. (2015) IP of resident AM fungi in soybean rotation involving maize in conservation tillage was found to be highly correlated (r=0.96 to 0.99) with grain yield of soybean and maintaining higher

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organic carbon which indicates the functioning of resident AM fungi in enhancing the soybean yield.

Rice (*Oryza sativa* L.) and soybean (*Glycine Max* (L.) Merr.) crops were grown in 2013-2016 to determine effect of drip irrigation in the different variants water levels on growth and productivity under conditions of the south-east of Kazakhstan.

Material and Methods

To solve objective solutions were carried out one-factorial and multiple-factoral field experiments on a demonstration site of the Center of distribution "Ushkonyr" of the Kazakh scientific research institute of agriculture and plant growing (*Figure 1*, *Tables 1-4*).

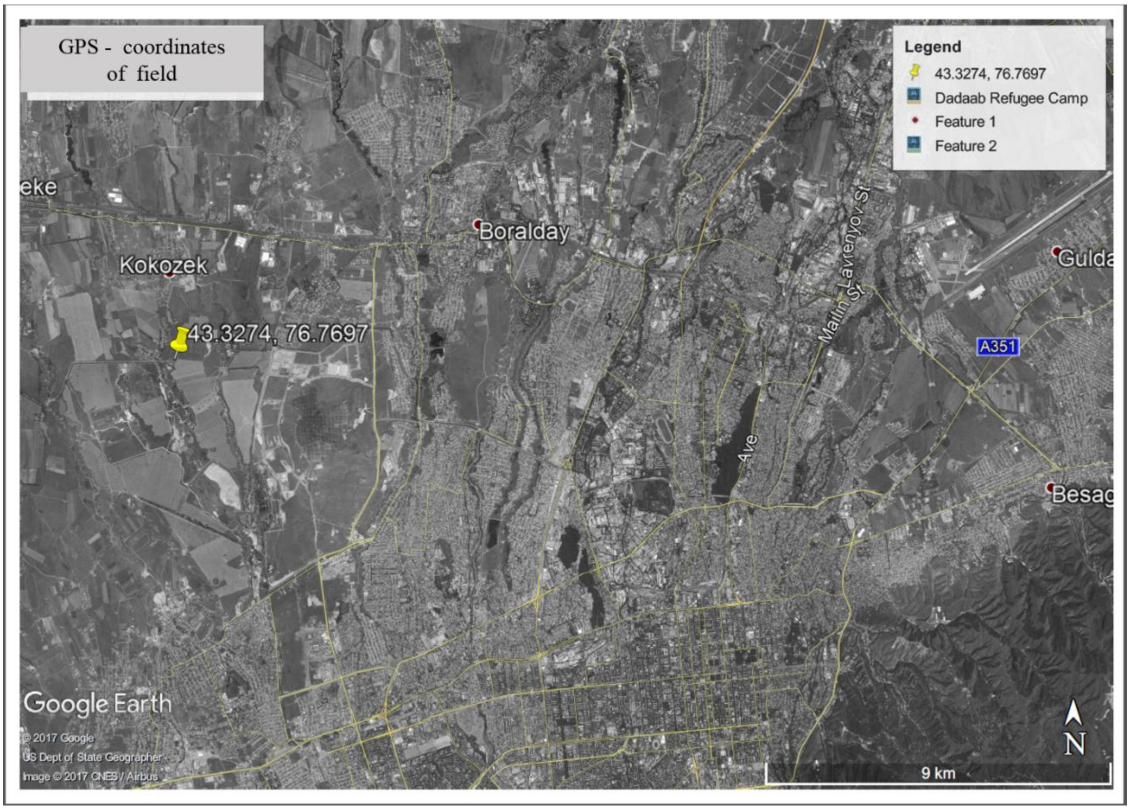

Figure 1. *Map of research area*

Experiment schemes

Experiment 1

The total area of the experiment is 0,15 hectares. The area of soil lots is 40-50 sq.m, replication is three staged. A fertilizer ground - $N_{120}P_{45}$.

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***Table 1.** Studying of ways of irrigation and rice seeding*

Ways of irrigation	Ways of rice seeding
1.Irrigation of rice by flooding	1. Drill seeding of rice
2.Surface drip irrigation with mulching film	2. A two-row tape seeding 30x70 cm with location of irrigated tape between lines. Distance between the irrigation tapes is 100 cm.
3.Subsurface irrigation with mulching film	3. A four-row tape seeding 10x20 cm with location of irrigated tape between lines. Distance between the irrigation tapes is 120 cm.

Experiment 2

The total area of the experiment is 0.6 hectares. The area of soil lots is 14 sq.m, replication is three staged. A fertilizer ground - $N_{120}P_{45.}$

***Table 2.** Studying of the rice irrigation mode*

Irrigation time	Irrigation day norm, m^3/ha	Expense of one dripper, l/hour
In the morning	20	0,5
In the afternoon	40	1,0
In the evening	60	1,5
At night	80	2,0

Experiment 3

The total area of the experiment is 0.04 hectares. The area of soil lots is 14 sq.m, replication is three staged.

***Table 3.** Studying of ways, doses and terms of introduction of mineral fertilizers under rice drip irrigation*

Doses of nitrogen fertilizers, kg of rate of application on 1 hectare	Doses of phosphoric fertilizers, kg of rate of application on 1 hectare		
	0	P45	P90
N0			
N30+N30			
N30+N30+N30			
N30+N30+N30+N30			
N30+N30+N30+N30+N30			

Experiment 4

The total area of the experiment is 0.04 hectares. The area of soil lots is 40-50 sq.m, replication is three staged. A fertilizer ground - $N_{120}P_{45.}$

***Table 4.** Studying of reaction of rice grades under drip irrigation*

Bakanas *s*	Marzhan	Regul	Xinjang 13-1834
Aru	Yantar	Leader	
Kazniizr 5	Kaz R-5	Xinjang 13-7013	

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The minimum areas of soil lots are connected with system of drip irrigation in experiments, high cost of materials and equipment.

Technique of carrying out accounts and supervision

- Phenological supervision over growth and development of plants in the main phases by a technique of State commission on a gradetesting;
- Studying of water and physical and meliorative properties of the soil. The accounting of field viability of seeds by calculation on 4 fixed platforms on 0.25 sq.m on all soil lots at full shoots. The accounting of density of plants standing plants by calculation of quantity of plants with 1 sq.m of a soil lot at the beginning and the end of vegetation of the studied cultures in two-staged replication.
- The accounting of dynamics of accumulation of biomass of plants of the studied cultures in the main phases of their development by selection of plants with 1 sq.m of each soil lot in two-staged replication with measurement of crude and dry weight.
- Water consumption of rice plants was defined by a balance method.
- The accounting of a contamination of crops, disease vulnerability and damageability by pests is carried out by the standard technique (Methodical instructions on monitoring of number of weed plants, pests and developments of diseases, Astana, 2004);
- Sampling for studying of maintenance of nutritious elements in the soil and plants will be carried out on the main growth phases and rice developments.
- Before harvesting from three replications of the experiment sheave samples with 1 sq.m were selected. Basic elements of structure of a rice harvest (quantity of plants, quantity of the general and productive stalks, mass of a sheaf, amount of grains in a whisk, the mass of 1000 grains) are determined by a technique of State commission on a gradetesting (Balashev, 1968);
- The accounting of a harvest is carried out seperately by the SAMPO-250 combine;
- Processing of harvest data according to Dospekhov's technique, 1985;
- Quality of grain by the technique stated in the reference book "Assessment of Quality of Grain", M. V.O "Agropromizdat", 1987;
- Studying of agrochemical properties of the soil by a technique of agrochemical researches. Calculation of economic efficiency is made on the actual costs of unit of the made production from area hectare according to the actual monetary and power costs of cultivation of the studied cultures.

Statistical processing of morphometric indicators was done via standard methods Lakin, 1990. The average number of M ± m was calculated by the Student method. The statistical processing of the data was carried out using Student's t-test for P> 0.05.

The system of drip irrigation includes: Irrigation water tank on 36 m^3 near the river Kaskelenka (*Figure 2*). The water pump Pedrollo F 32/200B with a productivity of 50 m^3/hour. A settler on 25 m^3 at the height of lower level of 5 m. Sandy strainer (*Figure 3*). Intake strainer; an injector for additional fertilizing; main pipes; distributive pipes; irrigation tapes with drippers for a surface drip irrigation.

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Figure 2. *Water intake system for drip irrigation*

Figure 3. *Water cleaning and additional fertilizing system*

For preparation of the soil under drip irrigation the general agrotechnology consisted in the following:

- Fall plowing on depth of 25-27 cm.
- Current planning.
- Early-spring braking.
- Introduction of phosphoric fertilizers for creation of grounds.
- Cultivation on depth of 12-15 cm.
- Preseeding processing on depth of crops of seeds.
- Crops of rice and soybean are made by a seeder 2BMJ-4 (People's Republic of China) which carries out simultaneous laying of a drip tape, a tension of the mulching film, seal of seeds over a film and a packing with filling of the soil (*Figures 4-5*).
- Norm of seeding- 15-30 kg/hectare depending on ways of crops.
- Additional fertilizing was made by ammonium nitrate with irrigation water of drip irrigation, schemes of the experiments: the first – at the beginning of tillering, the second - in a phase of a full tillering, the third – in a booting phase, the fourth – in an ear emergence phase, the fifth – in a phase of dairy grain ripeness.

Figure 4. *Double-row rice sowing with laying of both irrigation tape and tensioning of the film*

Figure 5. *Drip irrigation system*

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Harvesting was carried out by direct combining SAMPO-250 (*Figures 6-7*).

Figure 6. *Look of the experiment field after seeding*

Figure 7. *Plot costing of harvesting with a combine*

Agrophysical nature of the soil

Drip irrigation has positively influenced the maintenance of water-stable aggregate (>0,25 mm) in the above-mentioned soils under the studied cultures. So, their quantity from before drip irrigation and after that has increased in an arable layer of earth by 0.8-3.3%, at maximum increase under crops of soy without lamina with plowing on the 22-25 cm – by 3.3% and at minimum - under crops of oats with plowing on the 22-25th – by 0.8% and in subarable by 0.6-3.8% at the greatest under crops of also soy without lamina with plowing on the 22-25th – by 3.8% and the smallest - under crops of sugarbeet under lamina with disking on the 8-10th – by 0.6% (*Table 5*).

Definition and assessment of density of the light brown irrigated soils in May of reporting year demonstrates that the volume mass of 0-40 cm of a layer under various cultures before drip irrigation which can be accepted as initial fluctuated in the range of 1.22-1.29 g/cm^3. Indicators of volume mass of the specified layer of earth after carrying out a series of drip waterings have raised in September under the studied cultures up to 1.29-1.45 g/cm^3 (*Table 6*).

Table 5. *Agronomical valuable and water-stable aggregates in a foothill zone of Zailiysky Alatau, % of 2016*

Culture	Agronomical water-stable aggregates, %		Water-stable aggregates, %	
	0-20	20-40	0-20	20-40
1	2	3	4	5
May, 26				
Rice-1, plwng-22-25 cm	58	65	6.4	7.8
Rice -2, plwng-22-25 cm	53	61	6.9	8.6
Rice -3, plwng-22-25 cm	52	64	7.5	9.5
Rice under lamina,plwng-22-25 cm	52	55	8.3	10.6
Soy without lamina,plwng-22-25cm	51	67	8.4	8.8
Soy under lamina,plwng-22-25 cm	60	66	8.8	10.4
Sugarbeet under lamina,plwng-22-25 cm	55	63	6.9	9.4
Sugarbeet without lamina,plwng-22-25 cm	43	57	7.2	9.0

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Sugarbeet under lamina, untouched	59	65	7.2	8.1
Sugarbeet without lamina, untouched	44	64	7.5	10.1
Rice under lamina, untouched	61	64	5.4	6.4
Rice without lamina , untouched	52	60	7.4	10.3
September, 6				
Rice-1, plwng-22-25 cm	63	57	8.9	9.6
Rice-2, plwng-22-25 cm	65	60	9.2	10.5
Rice-3, plwng-22-25 cm	69	62	9.7	11.1
Rice under lamina,	68	61	10.2	11.2
Soy without lamina,	73	66	11.7	12.6
Soy under lamina,	72	65	10.9	12.8
Sugarbeet under lamina	70	64	8.4	11.2
Sugarbeet without lamina	69	59	9.0	10.8
Sugarbeet under lamina, untouched	68	60	8.3	8.7
Sugarbeet without lamina ,untouched	67	62	8.9	10.9
Rice under lamina, untouched	68	43	7.7	8.3
Rice without lamina, untouched	69	61	9.3	11.6

Table 6. *Changes of volume mass of the soil under the studied cultures in a foothill zone of Zaiylisky Alatau, g/m*^{3}

Culture	Tilling	Horizone				
		0-10	10-20	20-30	30-40	0-40
1	2	3	4	5	6	7
May, 26						
Rice without lamina	Moldboard plowing+ preseeding treatment	1.15	1.21	1.28	1.32	1.24
Rice under lamina		1.17	1.23	1.29	1.31	1.25
Soy without lamina		1.14	1.25	1.28	1.29	1.24
Soy under lamina		1.13	1.20	1.27	1.30	1.23
Sugarbeet under lamina		1.15	1.23	1.27	1.31	1.24
Sugarbeet without lamina		1.17	1.22	1.25	1.28	1.23
Sugarbeet under lamina	Preseeding treatment	1.18	1.25	1.32	1.29	1.26
Sugarbeet without lamina		1.23	1.27	1.30	1.32	1.28
Rice under lamina		1.22	1.26	1.33	1.35	1.29
Rice without lamina		1.20	1.25	1.30	1.33	1.27
September, 6						
Rice without lamina		1.23	1.30	1.39	1.44	1.34
Rice under lamina	Moldboard plowing+ preseeding treatment	1.24	1.28	1.37	1.43	1.33
Soy without lamina		1.27	1.48	1.46	1.51	1.43
Soy under lamina		1.30	1.50	1.49	1.51	1.45
Sugarbeet under lamina		1.25	1.28	1.35	1.40	1.32
Sugarbeet without lamina		1.20	1.26	1.34	1.35	1.29
Sugarbeet under lamina	Preseeding treatment	1.43	1.57	1.36	1.32	1.42
Sugarbeet without lamina		1.42	1.58	1.34	1.26	1.40
Rice under lamina		1.29	1.43	1.45	1.31	1.37
Rice without lamina		1.27	1.41	1.42	1.30	1.35

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Agrochemical nature of the soil (Table 7)

Table 7. *Key indicators of fertility of the soil of sugarbeet in the foothill irrigated zone of Zailiysky Alatau, 2016*

Ways of irrigation	Tilling	Depth, cm	Common humus, %	Labile humus, %	light hydrolyzed nitrogen, mg/kg	NO_3, mg/kg	P_2O_5, mg/kg	K_2O, mg/kg
1	2	3	4	5	6	7	8	9
May (26.05.)								
Ridge-and-furrow irrigation	Preseeding treatment	0-20	1.02	0.078	22.9	53	320	14.3
		20-40	1.20	0.104	21.4	67	333	12.9
Drip Irrigation		0-20	1.24	0.104	17.4	53	495	50.9
		20-40	1.22	0.143	19.1	50	391	23.5
Drip irrigation under lamina		0-20	1.04	0.143	14.1	50	428	26.7
		20-40	1.08	0.091	14.8	45	408	26.3
Ridge-and-furrow irrigation	Moldboard plowing+ preseeding treatment	0-20	1.34	0.195	7.90	50	462	23.5
		20-40	1.26	0.117	12.9	45	374	26.9
Drip irrigation		0-20	0.96	0.156	20.4	50	329	31.5
		20-40	1.12	0.130	19.5	50	304	12.9
Drip irrigation under lamina		0-20	1.04	0.091	15.1	45	308	80.0
		20-40	1.06	0.169	21.9	42	333	26.3
September (06.09.)								
Ridge -and-furrow irrigation	Preseeding treatment	0-20	1.43	0.196	7.80	45	423	33.6
		20-40	1.41	0.154	5.00	48	411	41.3
Drip irrigation		0-20	1.55	0.210	4.30	56	387	37.8
		20-40	1.41	0.168	6.60	50	375	27.1
Drip irrigation under lamina		0-20	1.20	0.224	10.0	70	403	38.9
		20-40	1.30	0.140	3.80	62	379	28.5
Ridge -and-furrow irrigation	Moldboard plowing+ preseeding treatment	0-20	1.43	0.126	22.4	70	394	18.2
		20-40	1.47	0.154	10.2	62	408	19.3
Drip irrigation		0-20	1.30	0.140	8.90	78	399	28.7
		20-40	1.37	0.112	12.9	84	408	19.0
Drip irrigation under lamina		0-20	1.28	0.140	3.80	56	261	48.3
		20-40	1.32	0.140	7.20	62	319	19.3

Results and Discussions

Ways of irrigation have significant effect on elements of structure of a rice crop. Drip irrigation with a two-row tape way of crops forms essentially distinct structure of a rice crop in comparison with usual technology of cultivation of rice. As it can be seen from data of *Table 1*, indicators of the general, and a productive tilling capacity of plants

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raises on 15.17-16.36 units or 7-8 times. At the same time the greatest productive tilling capacity (16.36) is reached under subsurface drip irrigation of rice (*Table 8*).

Table 8. *Structure of a rice harvest depending on ways of irrigation (average for 2013-2014)*

Ways of irrigation	Number of plants, piece/sq.m	Tilling capacity		Number of grains in a whisk,piece	Mass of 1000 grains, g	Proportion grain/ straw
		general	productive			
Flooding irrigation	106	2.11	1.86	39.1	32.5	0.24
Surface drip irrigation with mulching film	18	16.73	15.17	63.9	35.3	0.38
Subsurface drip irrigation with mulching film	17	18.89	16.36	62.0	34.7	0.38

There is a noticeable Influence of drip irrigation on formation of a bigger whisk grains/ear and mass of 1000 grains of rice, that eventually increased in a share of the productive part of a harvest expressed in the ratio 0.38 to 0.24 in control option.

Under drip irrigation depending on ways of crops formation of structural elements of a rice harvest was controversial (*Table 9*).

The greatest productive tilling capacity and mass of 1000 grains were formed by grades Bakanassky and Yantar, and whisk set of seed by Aru and KAZNIIR 5.

Table 9. *Structure of a harvest of rice grades depending on ways of crops under drip irrigation, 2013*

Grades	Ways of seeding	Number of plants, piece/sq. m	Tilling capacity		Number of grains in a whisk,piece	Mass of 1000 grains, g	Proportion grain/ straw
			general	productive			
Bakanassky	2-row	19	9.95	7.37	101	36.7	0.67
	4-row	32	4.94	3.06	84	35.1	0.52
Aru	2-row	18	10.4	6.61	108	33.9	0.68
	4-row	34	4.64	4.12	71	34.6	0.53
KAZNIIR 5	2-row	16	11.12	4.38	141	32.3	0.56
	4-row	32	5.94	2.66	94	30.8	0.35
Yantar	2-row	16	10.44	7.19	100	34.2	0.67
	4-row	30	4.26	3.22	85	35.4	0.42

Increase in irrigating norm of drip irrigation exerts positive impact on formation of rice tilling capacity and mass of 1000 grains (*Table 10*).

The irrigating norm of drip irrigation of 4000-6000 m3/hectare was the most rational norm of watering for formation of the largest sizes of structure of a rice harvest. In these conditions the productive tilling capacity has reached 11.75 pieces, and amount of grains in a whisk of 78 pieces.

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At the same time the best time of implementation of drip irrigation showed watering in the afternoon and in the evening. Positive impact of afternoon and evening terms of drip irrigation on an indicator of the general and productive tilling capacity is noted (*Table 11*).

Table 10. *Structure of a rice harvest depending on the mode of drip irrigation (average for 2013-2014)*

Irrigating norm m^3/hectare	Number of plants, piece/sq.m	Tilling capacity		Number of grains in a whisk, piece	Mass of 1000 grains, g	Proportion grain/ straw
		general	productive			
2000	17	12.36	9.77	66.5	35.7	0.38
4000	15	13.43	10.40	78.0	34.2	0.35
6000	16	13.94	11.75	71.0	35.3	0.33
8000	18	11.45	8.73	67.7	36.4	0.36

Table 11. *Structure of a rice harvest depending on time of drip irrigation (2013-2014)*

Irrigating norm m3/hectare	Number of plants, piece/sq.m	Tilling capacity		Number of grains in a whisk,piece	Mass of 1000 grains, g	Proportion grain/ straw
		general	productive			
In the morning	17	11.24	9.65	70.4	37.0	0.36
In the afternoon	17	13.00	10.83	76.9	36.4	0.37
In the evening	18	12.67	10.39	73.5	35.5	0.32
At night	17	14.12	11.95	71.8	35.6	0.39

Significant effect on formation of structure of a harvest renders conditions of mineral food of rice. As it can be seen from *Table 12*, without fertilizers the productive tilling capacity of plants of rice under drip irrigation did not exceed 9 units.

Use of nitrogen fertilizers in additional fertilizing with irrigation water promotes sharp increase of coefficient of rice tillering, the indicator of the tilling capacity in option with four and five nitric additional fertilizing on ground P90 reaches size of 18.2-18.7 and a productive tilling capacity of 15.7-17.0 units.

Table 12. *Structure of a rice harvest depending on conditions of mineral food under drip irrigation (average for 2013-2014)*

Fertilizer	Number of plants, piece/sq.m	Tilling capacity		Number of grains in a whisk,piece	Mass of 1000 grains, g	Proportion grain/ straw
		general	productive			
No fertilizing	15	11.5	8.2	57.5	35.5	0.47
N30+N30	17	11.9	9.8	68.7	34.9	0.51
N30+N30+N30	17	13.0	11.5	61.5	34.9	0.37
N30+N30+N30+N30	15	17.9	15.9	58.4	35.1	0.38
N30+N30+N30+N30+N30	19	14.6	13.2	60.3	36.1	0.36
P45	17	9.8	8.5	80.9	34.0	0.65
Ground1+ N30+N30	16	14.6	12.0	65.6	35.1	0.46

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Ground1+ N30+N30+N30	16	15.6	11.4	68.0	36.1	0.45
Ground1+ N30+N30+N30+N30	16	15.3	13.1	55.3	36.6	0.37
Ground 1+ N30+N30+N30+N30+N30	18	13.39	11.1	92.2	33.1	0.39
P90 - Ground 2	18	13.2	11.8	51.3	35.7	0.29
Ground 2+ N30+N30	19	15.4	11.1	55.9	35.5	0.33
Ground 2+ N30+N30+N30	17	15.0	13.2	61.1	33.2	0.34
Ground 2+ N30+N30+N30+N30	17	18.2	15.7	66.7	37.0	0.38
Ground 2+ N30+N30+N30+N30+N30	18	18.7	17.0	63.7	33.6	0.39

Results of the accounting of a harvest of the first year of researches (*Table 13*) show that in option with a usual way of an irrigation with flooding with introduction of average doses of fertilizers the harvest of rice of 29.1 c/hectare are received. In options on studying of drip irrigation ways without the mulching film crops of rice were unaccountable, have grown with weeds and at a booting phase growth of plants has stopped. Mechanical and chemical ways of control of weeds in these options are not effective. Therefore, in the next years options of drip irrigation without the mulching film are excluded from experience.

Table 13. *Crop yield of rice depending on ways of irrigation, c/hectare*

Ways of irrigation	2012	2013	2014	Average
Flooding irrigation	29.1	17.4	32.2	26.2
Surface drip irrigation	0	-	-	-
Surface irrigation with film	48.5	49.3	59.4	52.4
Subsurface drip irrigation	0	-	-	-
Subsurface drip irrigation with film	20.3	58.6	50.8	43.2
P=4.3%	3.6	4.5	4.9	
HCP 0.95=5.1 centners per a hectare	2.9	5.6	7.0	

Efficiency of drip irrigation ways by years of researches was ambiguous. In 2012 and 2013 there was an efficiency of surface way of drip irrigation, and in 2013 - subsurface drip irrigation with mulching film. In general, in three years at a usual way of cultivation of rice by flooding 26.2 c/hectare had been received, and at ways of drip irrigation with a film 43.2-52.4 c/hectare of a grain yield of rice are received. The greatest and most stable grain yields of rice by years have been received under surface drip irrigation with use of the mulching film.

In 2013 there were formed experiment options with 4-row tape way of seeding. Results of the experiment show that the new way of seeding had no advantage comparing with 2-row tape seeding. As it is seen from data of *Table 14*, the new way of seeding was effective only under drip irrigation of an early ripe grade Aru. From the studied grades the greatest productivity in Almaty region showed the grade Bakanassky under drip irrigation with 2-row tape seeding.

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Table 14. *Productivity of grades of rice depending on ways of crops under drip irrigation, c/hectare*

Сорта	Ways of seeding		
	2-row tape		4-row tape
	2012	2013	2013
Bakanassky	48.5	51.2	28,7
Aru	42.1	38.7	35,8
KAZNIIR 5	46.6	34.4	21,3
Yantar	33.5	35.5	26,8
P= 4.9%	3.4	6.4	
HCP 0.95=4.0, centners per a hectare	2.6	5.4	

Studying of the mode of rice drip irrigation shows that relatively big crops of rice are received when watering by irrigation norm 4000-6000 m3/hectare (*Table 15*) in the afternoon and in the evening.

Relatively low productivity of rice at the morning term of drip irrigation can be explained by low temperature of irrigation water. Whereas at the afternoon term of watering, water significantly heats up when passing through a drip tape, at evening watering irrigation water heats up in the tank.

Table 15. *Productivity of rice depending on the mode of drip irrigation, c/hectare*

Irrigating norm, m^3/ha	Irrigation time			
	morning	afternoon	evening	night
2013				
2000	39.2	39.9	42.6	46.8
4000	43.6	43.3	39.2	47.6
6000	43.8	40.0	46.3	50.0
8000	38.7	39.2	42.4	47.1
P = 4.7%, $HCP_{0.95}$ = 4.9 c/ha				
2014				
2000	27.1	34.6	37.6	36.2
4000	28.5	55.0	57.1	43.0
6000	34.7	53.8	60.9	57.0
8000	34.8	50.8	58.7	48.0
P = 5.4%, $HCP_{0.95}$ = 5.8 c/ha				
Average for 2013-2014				
2000	33.2	37.3	40.1	41.5
4000	36.1	49.2	48.2	45.3
6000	39.3	46.9	53.6	53.5
8000	36.8	45.0	50.6	47.6

Studying of efficiency of nitric additional fertilizing under drip irrigation on three phosphoric food (*Table 16*) shows that without introduction of phosphoric fertilizers of an increase of a harvest from nitric additional fertilizing makes 6.6-13.1 c/hectare. On this background two additional fertilizing by drip irrigation on 30 kg on hectare are rational and the harvest reaches 46.2 c/hectare.

Efficiency of nitrogen fertilizers was most accurately shown in 2014, when additional fertilizing from nitric have made 16.6-36.9 c/hectare depending on a phosphoric ground and number of nitric additional fertilizing. At the same time nitrogen share factor in formation of a harvest has made 70%, to 4% of phosphorus share.

Table 16. *Productivity of rice depending on conditions of mineral food under drip irrigation, c/hectare*

Level of nitric feeding	Level of phosphoric feeding		
	0	**P45**	**P90**
2013			
N0	33.1	43.2	45.1
N30+N30	46.2	42.5	55.8
N30+N30+N30	41.8	54.6	55.4
N30+N30+N30+N30	39.7	44.6	44.5
N30+N30+N30+N30+N30	39.8	38.0	46.0
P = 4.4 %, $HCP_{0.95}$ = 4.7 c/ha			
2014			
N0	23.5	40.8	37.2
N30+N30	36.0	42.0	35.9
N30+N30+N30	37.6	39.1	48.2
N30+N30+N30+N30	55.2	49.4	48.3
N30+N30+N30+N30+N30	58.2	57.4	74.1
P = 5.2 %, $HCP_{0.95}$ = 5.7 c/ha			
Average for 2013-2014			
N0	28.3	42.0	41.2
N30+N30	41.1	42.3	45.9
N30+N30+N30	39.7	46.9	51.8
N30+N30+N30+N30	47.5	47.0	46.4
N30+N30+N30+N30+N30	43.7	47.7	60.1

In the experience it has been shown that mulch has excellent impact on the growth and development of plants and has a negative impact on the nodule bacteria, that in the early phases provide favorable conditions for growth and development of soybean. In the variant without mulch, which are actively developing nodule bacteria in the initial phase inhibit the growth and development of soybeans, in connection can be noted in the early phases of nodule bacteria are parasitic.

In the literature, there are studies that confirm our data, but we note that in the version with a modern film developing nodule bacteria, but then their parasitism is not particularly stands out since, by the time the plant is well adapted in a supportive environment.

The observation showed that good loosening and sheeting occurs during branching, especially in the film version, the early spring foil retains not only moisture, but also allows heat in the field sites to clearly demonstrate good growth in the embodiment with the film, this proves a favorable film. The effect at drip irrigation, drip irrigation affects high productivity in comparison with traditional irrigation, since in experimental plots where the furrow is traditionally watered, soybean does not receive Due to the fact that water is not fully used in the direction, and here to save water. It is worth mentioning the procedure that should be implemented in all regions where there is a shortage of water, thus ensuring maximum productivity, not just soybean, And in many cultures.

In terms of the fact that water saving is due to the films also need to emphasize that the film under the water does not evaporate into the air and vaporized water is supplied to the reverse order of the plant. This not only saves water, but also keeps the heat under the film, which in the cold season supports the plants. In the Almaty region in the south-east of Kazakhstan the climate is not homogeneous, as the weather is variable, in this regard, the film is a necessity. In the *Figure 8* soybean were grown without mulch by drip irrigation. In the *Figures 9-10* soybean were grown with mulch under drip irrigation.

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Figure 8. *Soybean grown without mulch under drip irrigation*

Figure 9. *Soybean grown with mulch under drip irrigation*

Figure 10. *Growth and development of soybean under mulch*

Intensive growth and development of soybean plants during vegetation with formation of bigger biomass of plants, a leaf surface and basic elements of harvest structure eventually led to formation of more crops. It should be noted that the bigger soybean harvest was in option with surface drip irrigation on 3.6-9.2 c/hectare. Despite the bad weather conditions for growth and development of soy in the southeast of Kazakhstan, we have got a big crop (42.2 c/hectare) in option with use of the mulching

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film. At the same time the most effective way of drip irrigation was the surface one (Abdukadirova et al., 2016). As a result of research, there has been revealed the most acceptable option of cultivation and irrigation for the Kazakhstan soy gradessurface drip irrigation with the mulching film (*Table 17)*, and also the grades Lastochka and Dikovit showed the highest results in harvest in three years.

Table 17. *Structure of the harvest of soy grades under drip irrigation, 2013-2015*

Grades	**Amount of plants, piece/sq.m**	**Amount of branches, piece**	**Amount of beans in 1 plant, piece**	**Mass 1000 grains, g**	**Biological harvest, h/sq.m**
Zhalpaksay	20±2.2	8.2±0.9	52±14.5	146±0.9	467±19.8
Zhansaya	20±2.9	9.4±1.4	76±17.8	145±0.7	660±20.6
Lastochka	20±3.5	8.8±1.2	84±11.6	150±1.8	760±17.9
Vita	20±2.7	10.1±2.3	68±16.9	150±1.6	620±21.8
Dikovit	20±3.0	12.4±3.4	78±13.2	156±2.1	739±13.5

Three-year studies have been shown that the use of mulching film in rice and soybean cultures primarily contributes to the economic use of resources, as a result of which the growth of weeds is suspended. Since water helps stop the growth of weeds in rice and soybean, the use water saving technologies and mulching film when growing rice and soybean for increase productivity of rice and soybean in Kazakhstan.

For rice in southeastern Kazakhstan under drip irrigation there are 2-row ribbon cultures with a distance between the bands of 70 cm and rows in the tape of 30 cm. At the same time, the drop band is held within the slots in the belt. According to these results, it is recommended to increase the yield of rice to growth under the drip irrigation of mulch. Comparison of the productivity of soybean varieties showed that productivity in variants with mulch far exceeded variants without mulching.

Conclusions

Results of three years of researches on studying of rice and soybean drip irrigation efficiency let us make the following conclusions:

- Drip irrigation with application of the mulching film promotes emergence of early and amicable shoots of rice, intensive growth and development of plants, productivity increase.
- Drip irrigation with use of the mulching film at 8-10 times reduces a contamination of crops of rice that allows to exclude application of manual use, mechanical and chemical processing of crops.
- Use of the mulching film under rice drip irrigation promotes creation of the favorable nitric mode of the soil with big accumulation of ammoniac and nitrate nitrogen and mobilization of motile phosphorus.
- The best way of rice crops under drip irrigation is 2-row tape crops with distance between tapes of 70 cm and a row-spacing in a tape of 30 cm. At the same time the drip tape keeps within row-spacings in a tape.
- Most optimum mode for efficiency of rice was under rice drip irrigation in the evening and night period by irrigating norm of 4-6 thousand m^3/hectare. The consumption of irrigation water on cultivation of rice is reduced by 5-10 times.

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- Optimum conditions of mineral food of rice under drip irrigation of rice is reached by introduction of phosphoric fertilizers under preseeding cultivation by a dose of 45 kg on 1 hectare and 4-5 triple nitric additional fertilizing under drip irrigation to a phase of dairy ripeness of rice grain a dose of 30 kg of on 1 hectare. Efficiency of the introduced fertilizers significantly increases under drip irrigation.
- Drip irrigation with or without mulch was adopted for the experimental plots. The most effective way to combat weeds in soybean crops proved to drip irrigation under mulch film, where the number of weeds in the early growing season did not exceed 36-44 units / m^2.
- Despite the prevailing adverse weather conditions for the growth and development of soybean in the south-east of Kazakhstan, we have received a fairly high yield (42.2 tonnes / ha) on a variant using a mulching film.
- The results indicated that use mulch under drip irrigation significantly increase plant growth and production soybean. This increase is due to benefits such as increase in soil temperature, reduced weed pressure, moisture conservation, reduction of certain insect pests, higher crop yields, and more efficient use of soil nutrients.

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PROTECTIVE EFFECTS OF EXOGENOUS NITRIC OXIDE AGAINST LEAD TOXICITY IN LEMON BALM (*Melissa officinalis* L.)

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Abstract. This research investigated the effects of exogenous sodium nitroprusside (SNP) supplementation as nitric oxide (NO) on alleviating Pb-induced oxidative damage in lemon balm (*Melissa officinalis* L.) plants. Pb (100, 300 and 500 µM) alone and in combination with SNP (100 and 200 µM) were given to hydroponically grown *Melissa officinalis* L. plants. The findings suggested that in Pb-treated plants, height, total dry weight, and chlorophyll content of leaves markedly decreased while application of 100 µM SNP alleviated the inhibitory effect of Pb on plant growth and chlorophyll content. Pb exposure caused oxidative stress by elevating lipid hydroperoxide contents of the seedlings. Application of 100 µM SNP counteracted Pb toxicity by reducing lipid hydroperoxide contents of Pb-treated seedlings. Furthermore, it was found that the activities of antioxidant enzymes such as polyphenol oxidase, catalase, guaiacol peroxidase, and ascorbate peroxidase were increased in Pb-treated plants. The use of NO especially at low concentrations reversed Pb-induced negative effects whereas high concentrations of NO had no obvious alleviating effect on Pb toxicity in *Melissa officinalis* L. On the other hand, application of 100 µM SNP could function as a defense mechanism of the plant against Pb toxicity and mitigate Pb stress.
Keywords: *medicinal plant, oxidative sterss, lead pollution, antioxidant response, sodium nitroprusside*

Introduction

Heavy metal pollution has become a prominent environmental problem around the world. Among heavy metals, lead (Pb) is one of the most dangerous pollutants of the environment and Pb pollution in the air, water, and agricultural soil is an ecological concern due to its impact on human health and the environment. Although it is not an essential nutrient for plants, a great portion of Pb is easily taken up by plants from the soil and accumulated in roots while only a small fraction is translocated upward to the aerial parts of plant (Bai et al., 2015). Pb exposure affects growth and physiological parameters and leads to decreases in germination percent, length, and dry mass of roots and shoots, disturbing mineral nutrition and reducing cell division (Singh et al., 2011). In most studies, Pb has been known to induce oxidative stress through overproduction of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2), which in turn act on the unsaturated lipids in the cell membranes, ultimately leading to lipid peroxidation and damages in cell membranes (Kumar et al., 2013).

Plants are exposed to a wide range of pollutants, particularly heavy metals. However, they are different in their responses to heavy metal pollution. For instance, while some are sensitive to Pb, others show tolerance for this heavy metal and are able to take in considerable amounts of Pb (Nemati et al., 2013). In fact, plants have adopted

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specific ways to deal with pollution. Blocking the heavy metals' entrance into the cell via exclusion or binding them to cell wall is the first major mechanism of detoxification and this is an important mechanism for Pb pollution (Antosiewicz and Wierzbicka, 1999). Also, some plants have improved an anti-oxidative system, including anti-oxidative enzymes such as guaiacol peroxidase (GPOX), catalase (CAT), and ascorbate peroxidase (APX) (Cakmak and Horst, 1991).

Nitric oxide (NO), a free radical in living organisms, is considered a key signaling molecule and a phytohormone which has important roles in various physiological processes of plants such as germination, growth, senescence, photosynthesis, and response mechanisms to specific environmental stresses (Del Rio et al., 2004). It is reported to protect the plants against toxicity of reactive oxygen species (ROS), enhance their tolerance to abiotic stress, and improve their defense response (Besson-Bard et al., 2008; Neill et al., 2008). The application of a NO donor, SNP, confers tolerance to various abiotic stresses in plants by enhancing their antioxidant defense system under stress conditions (Xu et al., 2010). Nitroprusside plays its role through the production of compounds involved in detoxification of H_2O_2 and antioxidant enzymes such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (AXP), and guaiacol peroxidase (GPOX) (Tewari et al., 2006). NO has been found to reduce Pb uptake in Arabidopsis thaliana, thereby reducing toxicity symptoms (Phang et al., 2011). It also influences gene expression in response to oxidative stress in *Zea mays* leaves (Hermes et al., 2011). Nitroprusside decreased absorption of cadmium in *Melia azedarach* cuttings and improved some growth parameters under cadmium stress treatment (Arany et al., 2015). It was recently reported that exogenous application of SNP (as a NO donor) alleviated the hostile effects of abiotic stresses induced by heavy metals such as As (Hasanuzzaman and Fujita, 2013), Br and Al (Aftab et al., 2012) and Cu (Zhang et al., 2009). Pretreatment of cowpea seeds with SNP as NO donor before exposure to Pb had a protective effect against Pb toxicity conducing to an improvement of the chlorophyll value, RWC, and net photosynthetic rate by increasing antioxidant enzyme activities (Sadeghipour, 2015). *Melissa officinalis* L contains some phenolic and flavonoid compounds such as rosmarinic acid. Phenolic contents in plants have some antioxidant properties (Chen et al., 2001). This plant is widely used around the world because of its medicinal properties. In the *Melissa officinalis* L. plants treated with sodium nitroprusside, increase in concentrations of sodium nitroprusside led to an increase in oxidative stress molecules and malondialdehyde (Esmaeilzadeh et al., 2015).

Lemon balm (*Melissa officinalis* L.) is a member of the Lamiaceae family, which spreads widely from the western part of Europe (Ulbricht et al., 2005) to western and central parts of Iran. This plant is widely used around the world because of its medicinal properties. However, there is a paucity of data regarding the role of NO in alleviating Pb-induced toxicity. With that background in mind, we hypothesized that NO may ameliorate Pb-induced toxic effects in *Melissa officinalis* L. The present work aimed at investigating the role of exogenously supplied SNP (NO donor) in alleviating Pb stress in *Melissa officinalis* L.

Materials and Methods

Plant material and culture conditions

Melissa Officinalis L. seeds were cultured in perlite puts 2 centimeters deep at equal distances before they were sterilized with 5% sodium hypochlorite for 15 min and

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washed thoroughly with distilled water. The pots were irrigated by distilled water for 10 days. After germination, the seeds were nourished with Hoagland feeding solution for 15 days. Irrigation was continued during 3-leave stage with Hoagland solutions containing different concentrations of Pb $(NO_3)_2$ (0,100, 300, and 500 μM) and sodium nitroprusside (0,100, and 200 μM) alone and in combination for two weeks. Our experiment was performed under regulated conditions, daily temperature of 25/17° C and 60 ± 5% relative humidity. For the assessment of plant dry matter content, the plants were dried at 80° C for 48 h, to produce a constant weight. This research was conducted under the standard conditions at Sari Agricultural Research Center in Iran.

Determination of Photosynthetic pigments

The leaves were chopped into small pieces that were extracted with 80% acetone. The absorbance was measured at 645 nm and 663 nm for chlorophyll a and b, respectively. Then photosynthetic pigments (chlorophyll a and b) were assayed as per the method of Litehtenhaler and Wellburn (1983) formulae:

$$\text{Chl a (mg g}^{-1}\text{ leaf fresh weight)} = [12.7\ (OD663) - 2.69\ (OD645)] \times V/1000 \times W$$

$$\text{Chl b (mg g}^{-1}\text{ leaf fresh weight)} = [22.9\ (OD645) - 4.68\ (OD\ 663)] \times V/1000 \times W$$

$$\text{Total Chl (mg g}^{-1}\text{ leaf fresh weight)} = [20.2\ (OD645) - 8.02\ (OD\ 663)] \times V/1000 \times W$$

where:
OD= Optical Density
V = Volume of Sample
W = Weight of Sample

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring MDA, a major thiobarbituric acid reactive species (TBARS), and product of lipid peroxidation (Heath and Packer, 1968). Samples (0.2 g) were ground in 3 mL of trichloroacetic acid (0.1%, w/v). The homogenate was centrifuged at 10,000 g for 10 min and 1 mL of the supernatant fraction was mixed with 4 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The mixture was heated at 95° C for 30 min, chilled on ice, and then centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nM g^{-1} FW.

Determination of antioxidant enzymes

For extraction of antioxidative enzymes, shoots and roots were homogenized with 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.8) including 0.2 mM ethylene diamine tetra acetic acid (EDTA) and 2% insoluble polyvinyl pyrrolidone in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determining enzyme activities. The whole extraction procedure was carried out at 4 °C.

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PPO activity was determined by measuring the increase in the absorbance at 420 nm for catechol and 4-methylcatechol substrates and at 320 nm for pyrogallol substrate (Dogan et al., 2007). CAT activity was measured as the decrease in the absorbance at 240 nm due to the decline of extinguishment of H_2O_2 by the procedure of Patra et al. (1978). APX activity was measured by the decline in the absorbance at 290 nm, as ascorbate was oxidized (Nakano and Asada, 1981). GPX activity was calculated by using the extinction coefficient of 26.6 M^{-1} cm^{-1} for H_2O_2 at 436 nm and was expressed as nKat/mg^{-1} of protein. The 10 % gel was stained by the procedure of Hamill and Brewbaker (1969).

Statistical analysis

All data presented here are the mean values of three independent experiments with three replicates. All results were analyzed statistically by two-way ANOVA with SAS 9.1.3 software and means were compared with the LSD test ($P < 0.05$).

Results

Growth, malondialdehyde content and photosynthetic pigments

Total dry weight of Pb-treated plant was reduced significantly compared with control (*Table 1*). Different concentrations of Pb (0, 100, 300 and 500 µM) and interaction with sodium nitroprusside (100 and 200 µM) on plant growth, expressed that dry weight was increased in SNP100,however,200 µM SNP into Pb-treated solution was not diminished and dry weight of plant was decline significantly.

Effects of different concentrations of Pb (0, 100, 300 and 500 µM) and their interaction with sodium nitroprusside (100 and 200 µM) on plant growth expressed as height are shown in *Table 1*. Pb exposure inhibited the growth of *Melissa officinalis* significantly compared with control (Pb 0 Snp 0); however, this inhibition was moderated by the additions of 100 µM SNP. On the other hand, after application of 200 µM SNP into Pb-treated solution, Pb-induced inhibition on plant growth was not diminished and height of Pb-treated plant was reduced significantly (*Table 1*).

According to *Table 1*, the rate of MDA in shoots and roots increased in Pb-treated plants in comparison with the control group, significantly ($P<0.05$). Increasing MDA content resulted in increased lipid peroxidation in the metal-exposed plants. Under Pb stress, application of low SNP concentrations (SNP 100 µM) decreased MDA content, but increasing SNP concentrations (SNP200 µM) to Pb treatments did not alleviate the effects of Pb stress on lipid peroxidation (*Table 1*).

The rise in shoot chlorophyll a and b contents of *Melissa officinalis* plants exposed to Pb stress was statistically significant compared with control group; however, application of 100 µM SNP alleviated Pb toxicity in the photosynthetic system. High concentration of SNP (200 µM) had no mitigating effects on decreasing chlorophyll contents and both chlorophyll a and b contents were reduced, meaning fully compared to others (*Table 1*).

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Table 1. *Effects of different concentrations of SNP (0, 100 and 200 µM) on total dry weight, Chl. a and b, leaf and root MDA content, leaf and root H2O2 content in Melissa officinalis under Pb stress (0, 100, 300, and 500 µM).*

Pb	SNP	Height (cm)	Total dry weight (g)	Chl a (mg/g FW)	Chl b (mg/g FW)	Leaf MDA content (nmol.g-1.FW)	Root MDA content (nmol.g-1.FW)
0	0	22.85 a	4.6 b	2.803 ab	0.8 a	26.84 h	17.85 h
	100	22.25 ab	4.843 a	2.853 a	0.801 a	27.4 gh	17.96 h
	200	18.82 d	4.08 cd	2.61 bcd	0.767 b	33.77 de	21.44 g
100	0	21.58 bc	4.236 c	2.463 de	0.767 b	35.67 cd	25.43 f
	100	23.19 a	4.826 ab	2.706 abc	0.812 a	29.9 fg	21.61 g
	200	18.96 d	4.13 cd	2.346 e	0.757 b	37.72 c	26.94 ef
300	0	20.64 c	3.903 de	2.053 f	0.66 d	37.73 c	32.64 d
	100	22.29 ab	4.116 cd	2.496 cde	0.727 c	32.41 ef	28.3 e
	200	18.02 de	3.676 e	1.786 g	0.634 e	38.51 c	34.68 c
500	0	16.67 f	3.406 f	1.473 h	0.502 g	41.85 b	37.78 b
	100	17.79 e	3.686 e	2.056 f	0.581 f	37.5 c	32.98 cd
	200	16.1 f	3.34 f	1.266 h	0.453 h	46.81 a	41.41 a

Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).

Enzymes activity

Under Pb stress condition, CAT enzyme activity in shoots and roots of *Melissa officinalis* increased significantly (P≤0.05). In SNP-treated plant, both concentrations of nitroprusside increased catalase activity significantly (P≤ 0.05) and the increase was more pronounced at SNP100 concentration. Interaction between Pb and SNP ed that CAT enzyme activity increased significantly in Pb+SNP100 µM but there was a significant decrease at Pb+SNP200 µM. It seems that low concentration of NO enhances negative effects of Pb stress (*Figs. 1 and 2*).

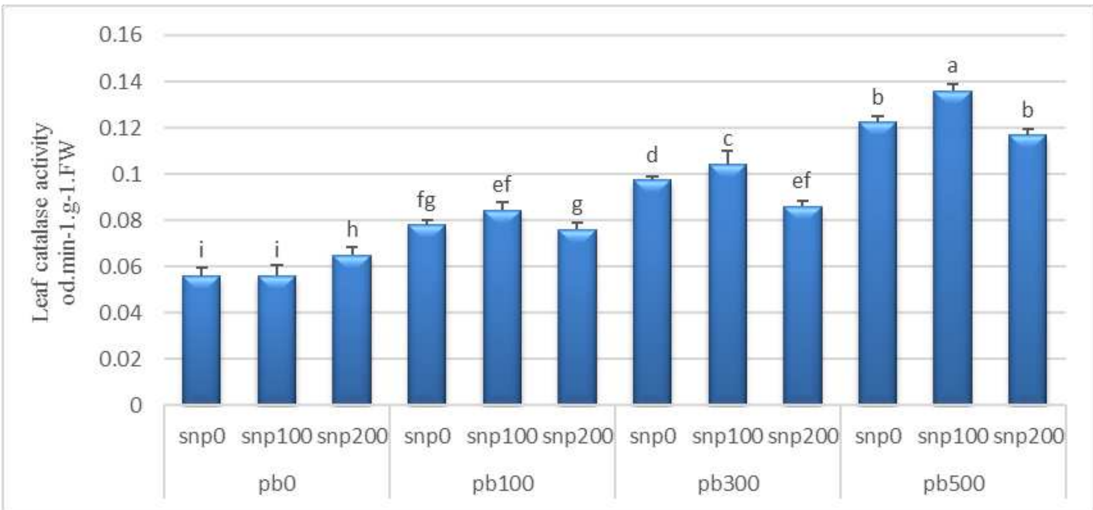

Figure 1. *Effects of different concentrations of SNP on catalase activity in shoots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

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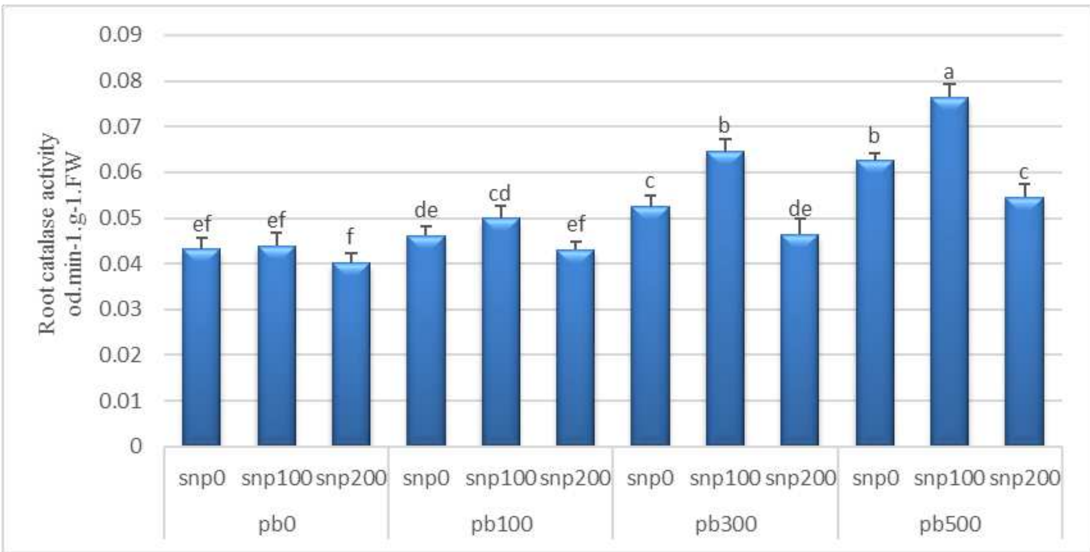

Figure 2. *Effects of different concentrations of SNP on catalase activity in roots of Melissa officinalis under Pb stress (P≤0.05). Means followed by the same letter are not significantly different (P ˂ 0.05) according to LSD test (n=3).*

With an increase in Pb concentrations, leaf APX enzyme activity decreased significantly (P≤0.05). Also, with increase in SNP concentrations, APX enzyme activity decreased. Under different concentrations of Pb and SNP, the maximum and minimum APX enzyme activities were recorded in Pb+SNP100 and Pb+SNP200, respectively (*Fig. 3*).

In roots, under Pb stress APX enzyme activity increased significantly (P≤0.05). In addition, in both roots and leaves, with increase in SNP concentrations, APX enzyme activity decreased. Also, APX enzyme activity significantly decreased (P≤0.05) in Pb+SNP200 treatment while there was a significant increase in the activity of this enzyme in the treatment containing Pb+SNP100 (*Fig. 4*).

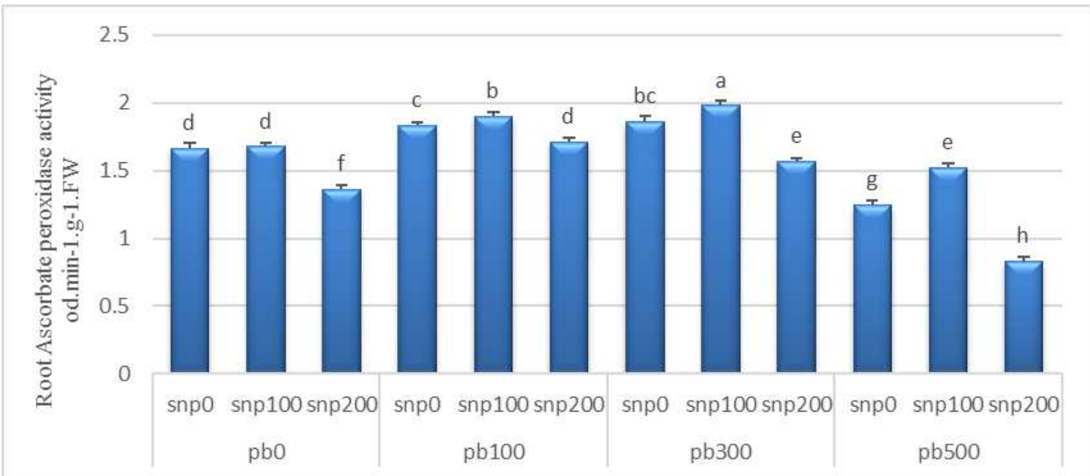

Figure 3. *Effects of different concentrations of SNP on ascorbate peroxidase in roots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P ˂ 0.05) according to LSD test (n=3).*

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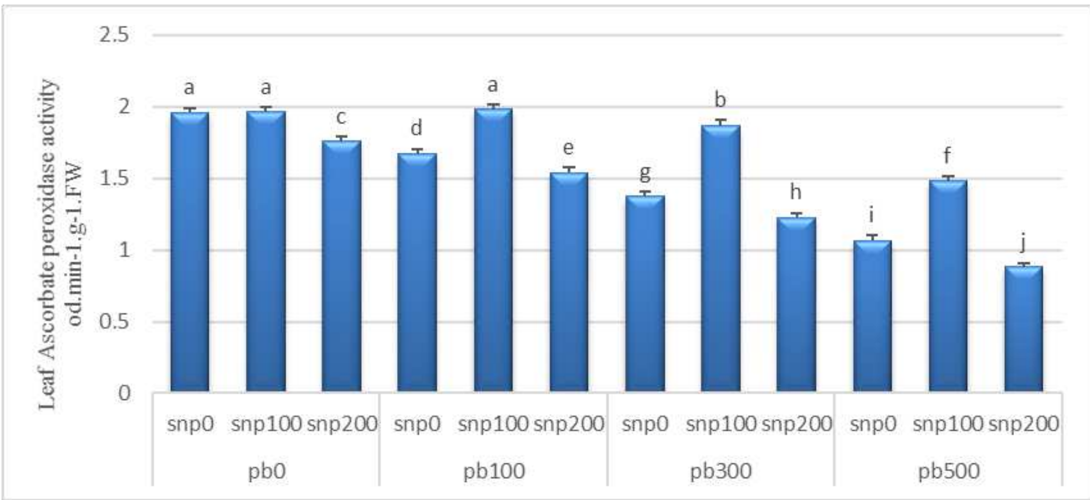

Figure 4. *Effects of different concentrations of SNP on ascorbate peroxidase in shoots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

In leaves of *Melissa officinalis* under Pb stress, guaiacol enzyme activity did not change with an increase in lead concentration but under SNP treatment, enzyme activity decreased significantly (P<0.05) with increasing SNP concentrations and in fact, SNP 100 µM showed the greatest increase in enzyme activity. The highest level of guaiacol activity was seen in the treatment containing Pb 300 µM + SNP100 µM.

In roost, under SNP concentration, enzyme activity was unaltered with increasing SNP. Also, with increasing concentrations of lead, enzyme activity increased in roots and the increase was particularly remarkable at Pb 300 µM. By application of SNP to Pb-treated plants, the highest level of guaiacol enzyme activity was seen at Pb (100 µM and 300 µM) + SNP100 µM while the lowest activity was seen at Pb 500 µM + SNP 200 µM (*Figs. 5 and 6*).

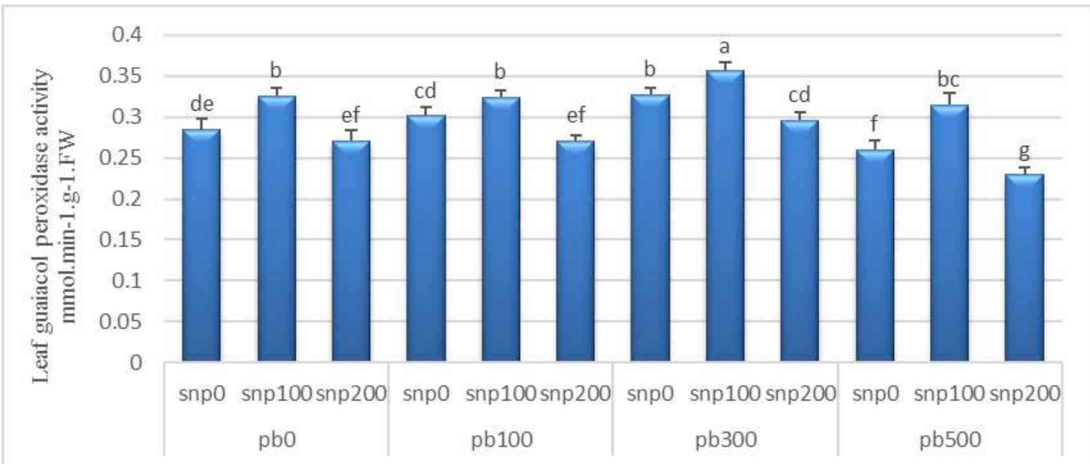

Figure 5. *Effects of different concentrations of SNP on guaiacol peroxidase activity in shoot of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

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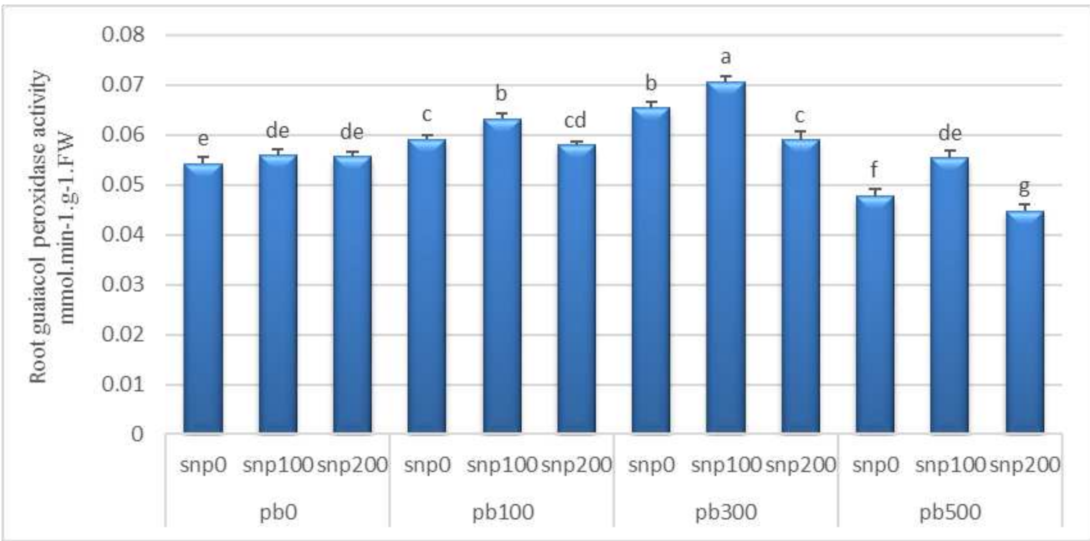

Figure 6. *Effects of different concentrations of SNP on guaiacol peroxidase activity in roots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

In leaves and roots of *Melissa officinalis* under Pb stress, polyphenol oxidase activity increase with increasing lead concentration except in Pb 500 µM. Under SNP concentrations, enzyme activity increased significantly ($P \leq 0.05$) with increasing SNP and SNP 100 µM showed the greatest increase in polyphenol oxidase activity. The highest level of polyphenol oxidase activity was seen at Pb (100 µM and 300 µM) + SNP 100 µM. Under SNP treatment, the highest level of polyphenol oxidase activity was seen at Pb (100 µM and 300 µM) + SNP 100 µM and the lowest level of activity was seen at Pb 500 µM + SNP 200 µM (*Figs. 7 and 8*).

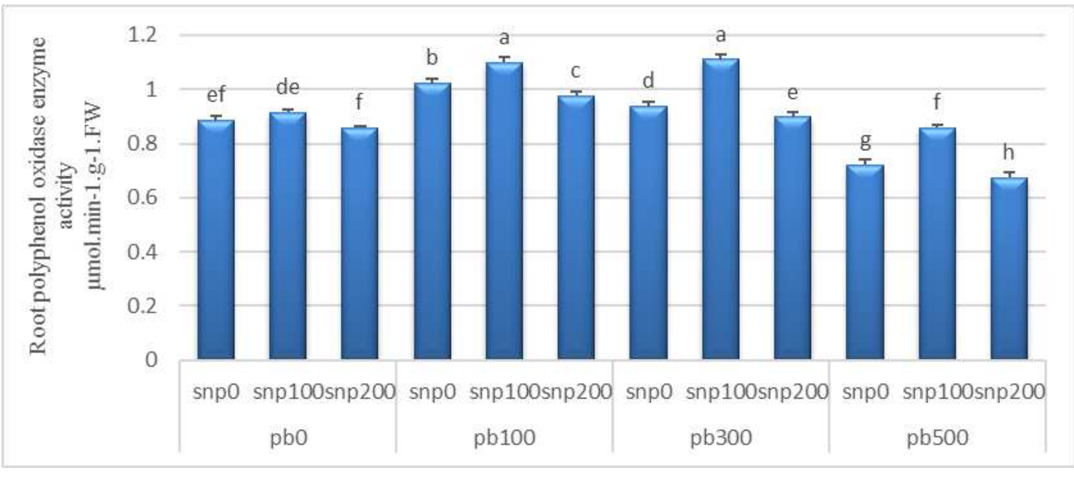

Figure 7. *Effects of different concentrations of SNP on polyphenol oxidase activity in roots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

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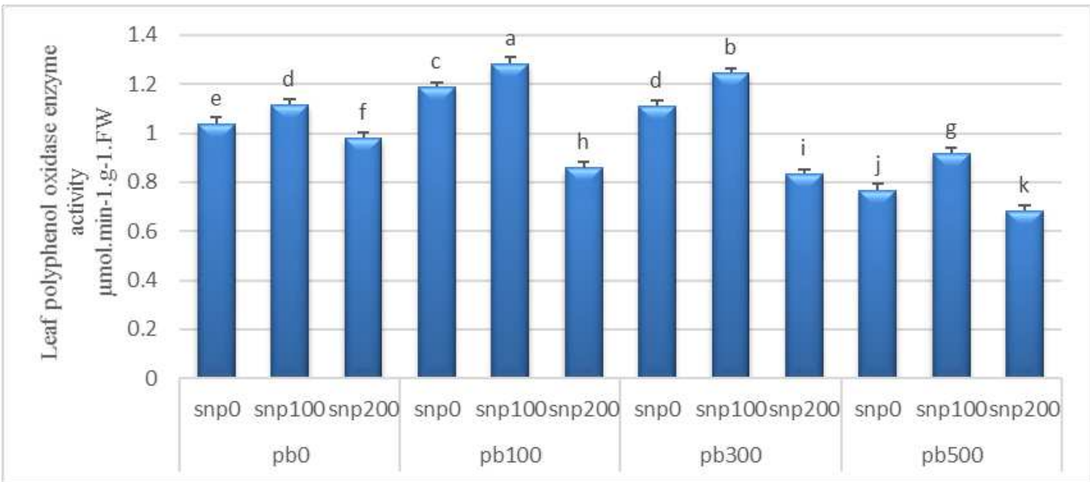

Figure 8. *Effects of different concentrations of SNP on polyphenol oxidase activity in shoots of Melissa officinalis under Pb stress. Means followed by the same letter are not significantly different (P < 0.05) according to LSD test (n=3).*

Discussion

Pb is a toxic heavy metal that exerts adverse effects on morphology and growth of plants causing inhibition of enzyme activities. It has been suggested that exogenous application of the NO donor, sodium nitroprusside (SNP), enhances plant tolerance to heavy metals (Kumari et al., 2010) and oxidative stress (Esim and Atici, 2013). In the present study, the probable effects of exogenous NO was investigated on reducing lead toxicity in *Melissa officinalis*. Different concentrations of NO were applied in the Pb-treated plants and the physiological parameters of *Melissa officinalis* under Pb Stress were investigated under different NO concentrations.

Plant biomass is a good indicator for characterizing the growth performance of plants in the presence of heavy metals. Results indicated that application of Pb (100, 300, and 500 µM) especially concentration of 500 µM decreased the dry weight of *Melissa officinalis*. However, simultaneous application of low concentration of NO (100 µM) increased the dry weight and height of *Melissa officinalis*. Pb-induced growth inhibition in this study was reflected by total dry weight. Similar response to lead treatment was previously reported in various plants (Brunet et al., 2009). Decrease in growth parameters (dry weight and height) in *Melissa officinalis* plants might be the result of changes in metabolic processes, e.g. oxidative damage, nutrient uptake, and photosynthesis or it might be associated with the inhibition of mitotic index observed under Pb heavy metal treatment (Sheldon and Menzies, 2005). Previous studies on the mechanism of Pb toxicity suggested that Pb binds to nucleic acids and causes aggregation and condensation of chromatin, inhibiting the process of replication and transcription and ultimately affecting cell division and plant growth (Johnson, 1998). In our study, Pb-induced inhibition was significantly reduced by the lower concentration of NO. The mitigation effect of lower concentration of NO might be because NO improved photosynthesis by increasing chlorophyll content and counteracted oxidative damage by decreasing the generation of ROS. When the higher NO concentration was applied in *Melissa officinalis* plants, the mitigation effect was not obvious.

In the present study, the chlorophyll content significantly decreased with the increasing concentrations of externally supplied Pb in *Melissa officinalis*. This reduction

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in chlorophyll content can be regarded as a specific response of the plants to metal stress, which resulted in chlorophyll degradation and inhibition of photosynthesis (Gajewska et al., 2006). Chlorophyll content showed maximum range at Pb (100 μM) and it was decreased beyond that concentration. Heavy metals inhibit chlorophyll and other pigments biosynthesis. Lead may impair the uptake of essential elements, such as Mg and Fe, for chlorophyll biosynthesis, substitute divalent cations, prevent enzyme activity like aminolevulinic acid dehydratase and increase chlorophyll biodegradation by enhancing chlorophyllase activity (Nareshkumar et al., 2014; John et al., 2009; Prasad and Prasad, 1987). Therefore, reducing the amount of chlorophyll can be due to several factors, namely, inhibition of enzymes associated with chlorophyll biosynthesis (John et al., 2009), inhibition of Calvin cycle enzyme activity (Sharma and Dubey, 2005), obstruction of electron transportation (Pourrut et al., 2011), and stomatal closure and distortion of chloroplast ultrastructure (Bharwana et al., 2014; Bai et al., 2015). Also, it may be the result of interaction of Pb with SH group of enzymes of chlorophyll biosynthesis as well as lipid peroxidation-mediated degradation as indicated by Singh et al. (2006). Similar cause was suggested by Tanyolac et al. (2007) in maize (*Zea mays* L.) under Cu stress.

Similarly, reduction in the chlorophyll content has been reported in many plant species exposed to Pb (Bharwana et al., 2014; Malar et al., 2014). Our results indicated that NO-mediated improvement of chlorophyll contents played a role in the enhancement of photosynthesis. In this experiment, exogenous NO at lower concentration (100 μM) increased the chlorophyll content under Pb toxicity but high concentration (200 μM) of NO had no positive effect and chlorophyll content significantly decreased at Pb (500 μM) ($P<0.05$). A high NO-induced accumulation of chlorophyll was also observed under Cd stress in Rapeseed and tomato (Jhanji et al., 2012) and wheat under As-induced oxidative stress (Hasanuzzaman and Fujita, 2013).

It was found that NO raised the antioxidant enzyme activity and consequently reduced chlorophyll desolation induced by ROS. On the other hand, NO also protected chlorophyll by increasing the uptake of Fe and Mg under Pb stress (Bai et al., 2015). In this way, NO protects chlorophyll by improving its biosynthesis and reducing its destruction under Pb stress conditions.

In our study, the MDA level was significantly increased by Pb treatment. Increase in the concentration of lead was observed to increase the level of malondialdehyde production. MDA is the final product of peroxidation of membrane lipids and accumulates when the plants are subjected to oxidative stress. Therefore, MDA level is routinely used as an index of lipid peroxidation under stress conditions. A high level of MDA is demonstrative of an increased formation of ROS and oxidative damage. Actually, ROS reject hydrogen from unsaturated fatty acids and produce lipid radicals and reactive aldehydes, which deform the lipid bilayer (Kumar et al., 2013). Pb is known to induce oxidative stress in plants due to the elevated manufacture of ROS (Singh et al., 2011). In this work, increasing MDA levels showed that oxidative stress and lipid peroxidation of membranes were induced by Pb stress in *Melissa officinalis* plants. This is supported by many studies, e.g., Pb toxicity caused oxidative damage and elevated MDA content in mung bean (Hassan and Mansoor, 2014), *elsholtzia argyi* (Islam et al., 2007), and cotton (Bharwana et al., 2014).

It was also observed in the present study that application of SNP 100 μM under Pb stress conditions inverted the Pb toxicity effect and decreased the MDA content compared to Pb treatment alone. However, the use of high concentrations of NO did not

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mitigate the oxidative effects of lead. NO moderated Pb stress in *Melissa officinalis* plants by averting oxidative damage via the enhancement of antioxidant enzyme activity. This suggests that NO acts as an efficient ROS scavenger and/or membrane stabilizer in *Melissa officinalis* plants subjected to Pb stress. The reaction of NO with ROS could prevent the damage to the membranes. It has been demonstrated that the reaction of NO with lipid alcoxyl (LO) and peroxyl (LOO-) radicals is quick (Beligni and Lamattina, 1999). Nitric oxide has also been reported to inhibit ion drip from plant tissue and protect plants against membrane damage due to lipid peroxidation under various stress conditions. This role of NO has been observed in heavy metal stress induced by Cd (Kumari et al., 2010), Br (Esim and Atici, 2013), and Al (Wang and Yang, 2005).

Under normal conditions, ROS are produced at a low concentration and there is a balance between the generation and alleviation of ROS. This balance may be disturbed by many environmental stresses. In order to protect themselves against oxidative stress, plants have developed a compound antioxidative protection system for scavenging ROS (Sharma et al., 2012). In order to scavenge ROS and to escape oxidative injury, plants possess an antioxidative system including antioxidative enzymes. Antioxidant system plays an important role in the plants' tolerance to stress conditions, which is based on the fact that the activity of one or more of these enzymes or antioxidant substances in general increases in plants exposed to stressful condition and this enhancement is related to increased stress tolerance (Fecht-Christoffers et al., 2003) and is considered as an efficient system for detoxification and cleaning up of the toxic oxygen species through an adaptive mechanism involving upregulation of anti-oxidative enzymes like SOD, CAT, PPO, APX, and GPX (Foyer et al., 1994).

Our results show that activities of enzymes had various effects on *Melissa officinalis* under Pb-stress. APX enzyme activity decreased with increasing Pb concentrations but CAT and PPO enzyme activities increased while GPX enzyme activity was unchanged compared to the controls. However, application of exogenous NO, particularly 100 μM improved antioxidant enzyme activity. Modification in the activity of antioxidative enzymes with Cd stress has been reported by a number of researchers (Mishra et al., 2008; Laspina et al., 2005).

Our results show that the maximum activity of CAT was found at Pb 500 μM and we found that with increasing concentration of the lead, activity of CAT increased. CAT is a generally present oxidoreductase that disintegrates H_2O_2 to water and molecular oxygen, and it is one of the key enzymes involved in the ejection of toxic peroxides. Increase in CAT activity can be illustrated by an increase in its substrate, to support the level of H_2O_2 as an adaptive mechanism of the plants (Reddy et al., 2005). Therefore, reduction in the activities of CAT might be due to the formation of a protein complex with metals that results in the structural integrity of proteins (Mohan et al., 1997). At higher concentration of Pb the activity of protein-based enzyme might be reduced due to the effect of ROS through reduced enzyme synthesis or change in assembly of its subunits (Verma and Dubey, 2003). CAT enzyme can play a role in controlling H_2O_2 level in cells. In subcellular compartments of pea root cells increased catalase activity was observed when plants were grown in nutrient $Pb(NO_3)_2$. However, a decline in the activity of catalase with an increased heavy metal concentration has also been observed in *Lemna gibba* (Parlak and Yilmaz, 2013) and in *Becopa monnera* (Mishra et al., 2006). Laspina et al. (2005) reported a decline in CAT activity under Cd stress. Thus, it

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seems that the differences in activities of antioxidant enzymes in heavy metal treated plants are highly dependent on species and experimental model.

Our results indicate an enhancement in the activity of GPX and PPO by the low concentration of Pb, suggesting that this enzymes work as an essential defense tool to resist Pb-induced oxidative damage in plants. GPX is located in cytosol, cell wall, vacuole, and in extracellular spaces. Increased peroxidase activity in Pb-stressed plants might be possibly due to increased release of peroxidases localized in the cell walls. Under sub lethal salinity and metal toxicity conditions, level of peroxidase activity has been used as potential biomarker to evaluate the intensity of stress. Similar to our findings, the activity of guaiacol peroxidase (GPX) increased in *S. Roxburghiana* plants exposed to Pb and in *Lemna minor* (Paczkowsk et al., 2007).

In the present study, APX activity was different in shoots and roots of *Melissa officinalis* under Pb stress. It was enhanced at low concentration of pb in roots, but, decreased in shoots. APX is generally attributed to an adaptive mechanism against increase levels of ROS content produced by Pb metal ions. Enzymes of ascorbate are localized mainly in chloroplasts and in other cellular organelles and cytoplasm, where they play an important role in combating oxidative stress. The positive correlation between APX activity and excess ROS may be attributed to effective scavenging of H_2O_2 content to protect stressed plants against oxidative damage induced under lead stress. Similar to the results observed in the present study, an increase in APX activity in plants following exposure of heavy metals was reported by Malar et al. (2014) in *water hyacinth*. In *Phaseolusvulgaris* and *Pisumsativum* (Rodriguuez-Serrano et al. 2006) and *Ceratophyllum demersum* (Mishra et al., 2008) APX reduced under Cd stress, but a decline in APX activity under Cd stress was reported by Gomes-Júnior et al. (2006).

Our results showed that Polyphenol Oxidase activity increased by the low concentration of Pb. Similar our study, Chinmayee et al. (2014) demonstrated that, PPO activity in *Jatropha curcas* L. increased in all plant parts under chromium, cadmium and lead stress. Also, Saffar et al. (2009) reported that in *Arabidopsis thaliana*, PPO activity might be the result of extended heavy metal stress. PPO catalyzes the formation of highly active Quinone that reacts with amino or sulfhydryl groups in proteins or enzymes. As a result, these reactions lead to changes in physical, chemical, or nutritional characteristics of proteins and, in many cases, to inactivation of enzymes including PPO (Mayer and Harel, 1979).

All the antioxidant enzymes studied in this work had maximum activity in shoots compared to roots. This might be due to moving of Pb in all parts as a micronutrient and this enhances the concentration of antioxidant enzymes in shoots compared to roots. In our study SNP (100 μM) was effective in improving all measured traits in the plants under lead stress but it had no positive effect in high concentration of Pb and this might be due to the fact that NO is itself a ROS and its dual behavior (protective or toxic) depends on the conditions (Beligni and Lamattina, 1999). The protective role is based on its capacity to regulate the level and toxicity of ROS. In many studies, the alleviation of oxidative damage by NO was attributed to the induction of activity of various ROS-scavenging enzymes (Esim and Atici, 2013). The present observation is in agreement with the previous findings on rice roots treated with As (Rodríguez-Serrano et al., 2006), and *Cassia tora* roots treated with Al (Kumari et al., 2010).

Two mechanisms have been reported which may explain NO protective action against oxidative damage. One possibility is that NO might activate antioxidant systems

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to scavenge ROS. Beside the role of NO in the activation of antioxidant enzymes that scavenge ROS, it can also react with $O_2 \bullet^-$ and generate peroxynitrite ($ONOO^-$). $ONOO^-$ is unstable and may be protonated and disintegrated to a nitrate anion and a proton, or it can react with H_2O_2 to produce a nitrite anion and oxygen (Fan et al., 2014). Second, it is now accepted that NO acts as a second messenger in plants. The cytoprotective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and to regulate the level and toxicity of ROS (Hayat et al., 2010). Present results indicate that SNP plays a protective role in APX, PPO, CAT, and GPX activity in *Melissa officinalis*. In the present study, the application of high concentration of NO did not reduce Pb-induced ROS damage. In fact it even produced more toxic effects in *Melissa officinalis.* One of the most intriguing behaviors in NO biology is its dual function as a potent oxidant and effective antioxidant. This dual role of NO might depend on its concentration as well as on the environmental conditions.

Conclusion

The present study demonstrated that Pb stress caused oxidative damage and membrane lipid peroxidation, leading to a significant decrease in chlorophyll content and shoot and root growth in *Melissa officinalis* plants. On the other hand, application of SNP as NO donor in *Melissa officinalis* under Pb stress had a protective effect against Pb toxicity by increasing antioxidant enzyme (PPO, CAT, APX, and GPX) activities leading to an improvement of the chlorophyll content and dry weight. Thus, exogenous NO can be used to alleviate Pb toxicity in Melissa. In fact, the lower concentration of NO (100 µM) had a higher protective effect on Pb toxicity, while high concentration of NO (200 µM) did not alleviate Pb toxicity considerably. NO may help plants to survive stressful conditions through its function as a signaling molecule in the activation of antioxidative enzymes or its direct reaction with active oxygen, nitrogen, and lipid radicals. While supplementation of SNP resulted in growth enhancement, as well as increase in the contents and activities of all investigated biochemical components. Thus, our results indicate that SNP application might alleviate Pb toxicities and regulate plant growth and development of *Melissa officinalis* and that Pb tolerance of *Melissa officinalis* cultivated in Pb-mediated nutrient solution increased at low concentrations of NO. This indicates that NO acts as an efficient ROS scavenger and membrane stabilizer in *Melissa officinalis* plants exposed to Pb stress.

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THE CORRELATION BETWEEN MANDIBULAR LENGTH VERSUS BODY MASS AND AGE IN THE EUROPEAN ROE DEER (*CAPREOLUS CAPREOLUS* L.)

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Abstract. This study investigated the correlation between mandibular length versus the age and body mass of European roe deer. Mandibular length was measured in 7560 roe deer (3888 females and 3672 fawns younger than 1 year) that were hunter-harvested in the Czech Republic in 2007-2012. The body mass and age of every animal were determined. Average mandibular length was determined at less than 130 mm in fawns and more than 153 mm in adult females. The greatest increase in mandibular length and body mass of 24.8 mm and 5.3 kg, respectively, was observed in animals aged up to 4 years. In older roe deer, mandibular length increased at a slower rate and remained fairly constant. Mandibular length increased with body mass, and the cross-correlation coefficient was determined at 0.8255. The results of this study indicate that mandibular length is a useful metric for describing the quality of roe deer populations and individuals.
Keywords: *craniometry, game management, individual development, morphometry, population*

Introduction

The European roe deer (*Capreolus capreolus*) is a species with high levels of morphological variation. Twenty-six subspecies of roe deer have been identified based on phenotypic and geographic variations in roe deer populations (Mayr, 1942; Sempéré et al., 1996). Differences in cranial measurements are significant determinants of inter-population variability in the species (Kulak and Wajdzik, 2009) and other wild cervids (Markov, 2014). Cranial dimensions are influenced by primary production which is responsible for the isolation of local populations and differences in cranial morphology. According to Stubbe and Passarge (1979) and Zedja and Koubek (1988), the body mass of animals is directly linked with habitat productivity which is influenced by soil type, vegetation, population density and ecotone length. Animals that forage on woody plants have shorter and wider crania than animals that feed mainly on herbaceous plants (Aragon et al., 1998). The mandible is one of the first bones in the body to ossify (Hewison et al., 1996), and mandibular length in adult animals is determined mainly by environmental conditions in early life.

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In wild animals, including game, the mandible constitutes interesting research material because it does not have any economic value. Analyses of mandibular measurements support the search for new parameters to describe variations in local populations and the quality of their habitats (Sheremetyeva and Sheremetyev, 2008; Hanzal et al., 2012; Mendoza et al., 2002).

The aim of this study was to investigate the relationship between mandibular length versus the age and body mass of roe deer.

Materials and Methods

The study was performed on mandibles of the European roe deer *Capreolus capreolus* that were hunter-harvested in the Žďár nad Sázavou District of the Czech Republic in 2007-2012. Mandibular length was measured in 7560 roe deer, including 3888 females (does) and 3672 individuals aged up to 1 year (fawns).

Roe deer were hunter harvested in the Žďár nad Sázavou District, Jihlava county of the Vysočina Region (Czech Republic) at the altitude of around 500 m above sea level. The district has an area of 1 579 km^2 (Misar et al., 1983). Water bodies occupy 2.9% of the district's area. Agricultural land and forests (where the animals were harvested) have a similar share of the district's area at around 49% and 41%, respectively (Czech Statistical Office, 2014).

Mandibular length (mm) was measured between the zygomatic arch and incisor root to the nearest 0.1 mm. The animals' body mass (kg) was determined to the nearest 0.1 mm, immediately after harvesting (in the hunting site) and before evisceration. Potential blood loss associated with hunter harvesting was not subtracted from body mass measurements.

The animals' age was estimated based on physiological features and the wear of mandibular teeth (Lochman, 1987; Vach, 1993). Due to the extensive experimental material (7560 mandibles), the age of the analyzed roe deer could not be determined with the use of laboratory methods for organizational reasons.

The following variables were processed statistically:

- body mass,
- mandibular length,
- age,
- harvest date.

The data were expressed as means ± standard error of the mean (SEM). The results were analyzed statistically by one-way ANOVA, and the significance of differences between groups was determined with Duncan's multiple range test at a significance level of $P \leq 0.05$. All calculations were carried out in the Statistica 10.0 program (StatSoft, 2011).

Results

Average mandibular length was determined at less than 130 mm in fawns and at more than 153 mm in adult females (*Table 1*).

The greatest increase in mandibular length and body mass of 24.8 mm and 5.3 kg, respectively, was observed in animals aged up to 4 years. In older animals, mandibular length increased at a slower rate and remained fairly constant (*Fig. 1*).

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***Table 1.** Average body mass (kg) and mandibular length (mm) in fawns and does*

Trait	Fawns (n=3672)	Does (n=3 888)
Average body mass	7.96	13.03
Average mandibular length	129.30	153.44

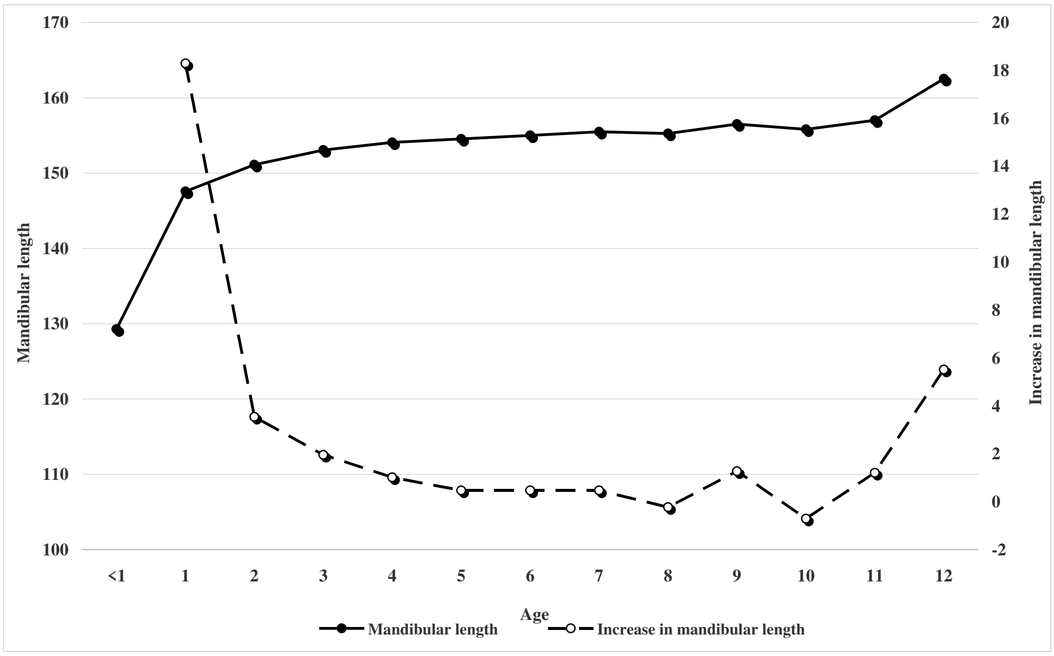

***Figure 1.** Average mandibular length [mm] and increase in mandibular length [mm] in roe deer aged to 12 years*

The greatest difference in mandibular length was observed between 2-year-old individuals and fawns, and it reached 18.28 mm on average (*Fig. 1*). The noted difference accounted for around 60% of the total increase in mandibular length in the analyzed period of life. The observed difference was highly significant (*Table 2*).

According to estimates, the youngest animals were approximately 5 months old, and the oldest individual was 12 years old in September of the first experimental year, which suggests that the overall increase in mandibular length in the examined period of life was approximately 30.4 mm.

Mandibular length increased with body mass (*Table 2*, *Fig. 1*), and the value of the cross-correlation coefficient reached 0.8255. It should be noted that body mass was characterized by greater variations than mandibular length across the examined age groups (*Table 2*).

In females, average mandibular length was determined at 153.44 mm, and this value was noted in hypothetical animals with average body mass of 13.03 kg, and average age of 4.4 years (*Table 1*). In fawns, average mandibular length reached 129.3 mm, and it was observed in hypothetical animals with average body mass of 7.96 kg, and average age of 5-7 months.

The average mandibular length of fawns harvested in each year of the study was analyzed (*Table 2, Fig. 2*). The measured parameter was higher in 2008, 2009 and 2012 than in the remaining years of the experiment.

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Table 2. *Mandibular length (ML) [mm] and body mass (BM) [kg] in differently aged [year] roe deer hunter-harvested in 2007-2012 (mean± SD) A, B, C, D - p ≤0,01; a, b, c, d – p ≤0,05*

	Age	**<1**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2007	**N**	588	62	67	57	58	56	62	63	69	60	76	-	-
	ML	129.02A ±3.27	147.22B ±3.18	151.10B ±4.01	152.34B ±4.16	154.21B ±4.46	154.91B ±4.56	154.88B ±4.62	155.41B ±4.89	156.31B ±4.93	154.42B ±4.86	157.21B ±4.92	-	-
	BM	8.3A ±0.90	11.0BC ±1.11	13.2B ±1.29	13.6B ±1.31	13.6B ±1.39	13.8B ±1.45	13.9B ±1.51	14.5B ±1.61	13.2B ±1.48	14.7B ±1.57	15.2BD ±1.44	-	-
2008	**N**	665	63	68	58	60	74	63	64	71	74	51	12	
	ML	130.41A ±3.11	149.42B ±3.15	151.61B ±3.99	153.04B ±4.12	154.41B ±4.55	154.15B ±4.67	154.51B ±4.57	155.21B ±4.68	154.57B ±4.87	155.68B ±4.91	155.32B ±4.86	157.09B ±4.91	-
	BM	8.7A ±1.01	13.2B ±1.09	13.4B ±1.19	12.5B ±1.16	14.4B ±1.42	13.2B ±1.37	13.4B ±1.41	13.6B ±1.48	13.4B ±1.42	11.6B ±1.28	12.7B ±1.31	13.7B ±1.50	-
2009	**N**	597	61	58	55	75	56	61	63	73	59	75	7	
	ML	130.44A ±2.99	144.12B ±3.10	152.08B ±4.11	154.22B ±4.21	154.56B ±4.99	155.71B ±4.66	156.48B ±4.68	156.49B ±4.57	156.82B ±4.69	156.69B ±4.88	157.37B ±4.99	154.98B ±4.82	-
	BM	8.8Aa ±0.99	11.2Bbc ±1.16	12.6B ±1.21	12.4B ±1.18	13.6Bd ±1.32	13.8Bd ±1.50	13.9Bd ±1.49	13.3Bd ±1.43	13.4Bd ±1.46	13.3Bd ±1.44	14.2Bd ±1.66	12.9B ±1.27	-
2010	**N**	641	57	60	55	61	59	69	61	68	70	69	-	7

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	ML	129.98A ±2.97	147.67B ±3.59	150.25B ±3.98	153.17B ±4.19	153.64B ±4.50	154.58B ±4.59	153.79B ±4.81	154.78B ±4.69	154.89B ±4.76	155.78B ±4.81	154.29B ±4.88	-	162.51B ±5.01
	BM	8.9Aa ±0.79	11.3bc ±1.08	12.5B ±1.30	13.1B ±1.41	13.4Bd ±1.38	13.8Bd ±1.47	13.2B ±1.39	13.9Bd ±1.48	14.1Bd ±1.58	14.2Bd ±1.63	12.3B ±1.32	-	12.5B ±1.36
2011	**N**	583	69	66	56	70	63	59	66	65	61	71	-	-
	ML	128.22A ±3.01	148.60B ±3.44	150.21B ±4.00	153.31B ±4.13	154.23B ±4.87	154.46B ±4.63	154.49B ±4.73	155.22B ±4.79	154.29B ±4.69	162.02B ±4.92	156.50B ±4.87	-	-
	BM	8.9A ±0.88	12.7 ±1.14	12.4 ±1.27	13.3 ±1.29	13.6 ±1.37	13.8 ±1.49	13.9 ±1.45	13.3 ±1.39	13.5 ±1.39	14.1 ±1.50	12.5 ±1.37	-	-
2012	**N**	598	69	71	58	68	66	68	70	62	70	73	-	-
	ML	129.51A ±2.79	145.62B ±3.27	152.21B ±4.08	152.64B ±4.17	154.44B ±4.94	154.39B ±4.60	156.22B ±4.70	156.40B ±4.69	154.91B ±4.83	157.62B ±4.85	156.89B ±4.93	-	-
	BM	8.9Aa ±1.03	10.5bCc ±1.11	12.7Bd ±1.19	12.6Bd ±1.20	13.1BD ±1.38	12.5Bd ±1.33	13.1BD ±1.47	13.1BD ±1.13	12.1Bd ±1.31	13.6BD ±1.38	12.7Bd ±1.40	-	-
2007-2012	**N**	3672	381	390	339	392	374	382	387	408	394	415	19	7
	ML	129.27A ±3.01	147.56B ±3.49	151.10B ±4.09	153.05B ±4.18	154.06B ±4.88	154.53B ±4.62	155.00B ±4.79	155.47B ±4.77	155.24B ±4.81	156.51B ±4.89	155.80B ±4.94	157.00B ±4.88	162.51B ±5.01
	BM	7.8A ±0.97	11.9Bc ±1.10	12.7B ±1.24	12.9B ±1.30	13.3Bcd ±1.41	13.2Bd ±1.49	13.2Bd ±1.52	13.4Bd ±1.48	13.3Bd ±1.50	13.3Bd ±1.48	13.2Bd ±1.52	13.3Bd ±1.36	12.5B ±1.36

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Individuals that were harvested later in the year (December) and were, therefore, older, were characterized by longer mandibles than fawns harvested in earlier months. Mandibular length was approximately 10.6 mm higher in roe deer harvested in December than in individuals harvested in September, and it was determined at 133.6 mm and 123.0 mm, respectively (*Table 3, Fig. 2*).

No significant differences in mandibular length were noted across the experimental years.

Table 3. *Mandibular length [mm] in fawns younger than 1 year in different months of the experimental years*

Month		Year					
		2007	2008	2009	2010	2011	2012
Sept	N	109	84	99	88	78	101
	mean±SD	122.6±2.92	123.6±2.61	124.4±2.55	123.0±2.99	121.4±2.74	123.4±2.77
Oct	N	190	213	184	115	99	126
	mean±SD	127.3±2.61	127.3±2.66	128.5±2.99	126.6±3.01	126.0±3.22	126.4±2.97
Nov	N	211	194	174	234	168	184
	mean±SD	130.3±2.97	131.8±2.99	131.5±2.99	131.4±3.10	131.2±2.98	132.2±3.12
Dec	N	78	174	149	195	238	187
	mean±SD	132.7±3.11	133.9±3.10	134.1±3.05	133.2±3.12	133.5±2.99	134.3±2.99

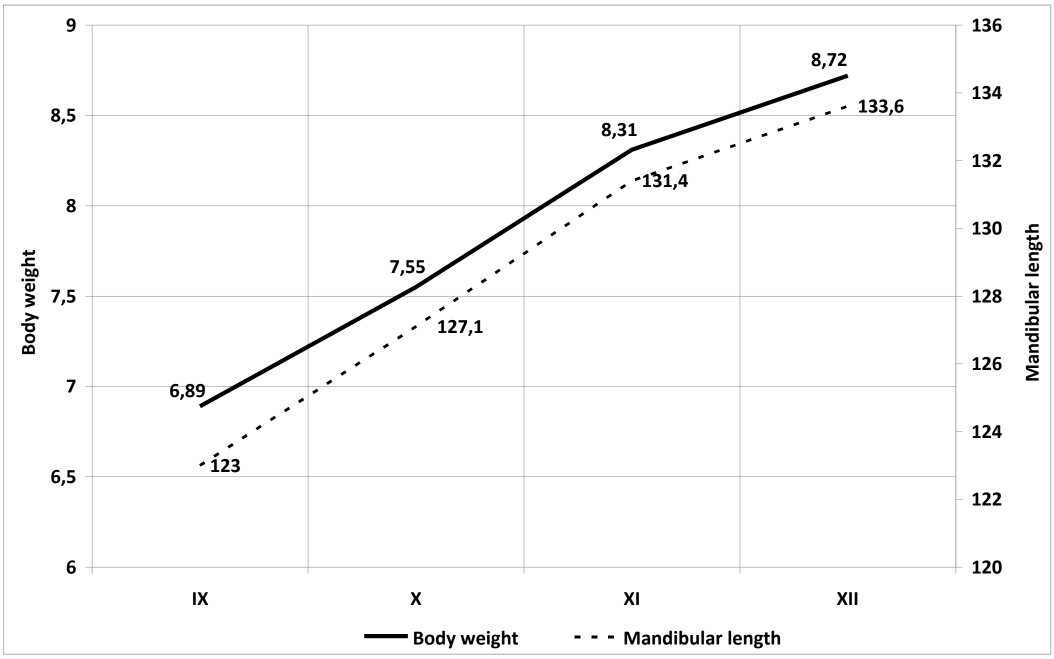

Figure 2. *Average body mass [kg] and mandibular length [mm] in one-year-old roe deer fawns hunter-harvested in different months of the year*

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Discussion

In animals, selected elements of the skeletal system are often used as retrospective indices to describe bodily dimensions, physiological development and resistance to seasonal changes in food availability (Zanneśe et al., 2006b). Slow bone growth observed under unfavorable environmental conditions can be compensated for in periods when food is more abundant (Bailey, 1984). Animal density, habitat quality, genetic structure and climate are significant predictors of body size in ungulates. Mandibular length can be an important indicator of physiological status in many cervid species. Animals living in more supportive environments are characterized by more developed maxillary and mandibular bones (Høye and Forchhammer, 2006).

Wustinger et al. (2005) analyzed 29 mandibles of female roe deer from the Polish region of Wielkopolska. Mandibular length was determined at around 132 mm in fawns and 156 mm in adult individuals. In our study, the examined mandibles were shorter at 129.3 mm in fawns and 153.4 mm in adult females. The results reported by Wustinger et al. (2005) could indicate that habitat conditions in the examined region were more favorable for roe deer.

In our study, average mandibular length in female roe deer from Czech lowland regions was more than 2 cm lower than that noted in does inhabiting the Prokletije mountain range in Serbia, where this parameter was determined at 156.55 mm (Labus et al., 2010). No significant differences in mandibular length were observed between males and females. The average mandibular length of roe deer in Bosnia and Herzegovina (not adjusted for gender or age) was also determined at 156 mm (Avdić et al., 2013), which suggests that the above value is typical of *Capreolus capreolus* inhabiting the Balkan region.

According to Zanneśe et al. (2006a) and Dvorak et al. (2002), the highest rate of mandibular growth is observed in the first 5 years of life. Similar observations were made in our study of roe deer from the Czech Republic. Mandibular length increases most dynamically in the first year of life, after which, this parameter increases annually by around 1 cm on average until the age of 4 years. Similar results were reported by Vach (1993) who observed the highest rate of mandibular growth in roe deer in the first 2 years of life.

An analysis of an increase in mandibular length in all examined individuals (aged 1 to 12 years) yielded similar results to those reported by Anděra & Horácék (1982) in whose study, the evaluated parameter increased from 142 mm to 164 mm. In our study, average mandibular length in all roe deer was determined at 151.71 mm, and it increased from 129.27 mm in fawns to 147.56 mm in one-year-olds to 162.51 mm in 12-year-olds. According to Hrabe and Koubek (1991), the greatest increase in cranial length is observed between 11 and 39 months of age.

In does, mandibular growth is completed at 4 years of age. In cervids, this parameter is correlated with other, apparently unrelated physiological functions, such as fertility. Animals with better nutritional status and higher body mass reach puberty earlier, and the probability of ovulation in does exceeds 0.95 when mandibular length reaches 130 mm. The population of one-year-old females that had calved was higher in habitats where food was more available. In does aged 1 to 7 years, age, body mass and mandibular length were significantly correlated with fertility. Fertility was most highly correlated with mandibular length (Górecki et al., 2014; Bertouille and Cromburugghe, 2002).

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A study investigating the influence of environmental factors on the mandibular length of roe deer in the Italian region of Belluno produced highly interesting results. Differences in mandibular length were determined in animals inhabiting northern and southern parts of the region. The studied locations are marked by considerable differences in altitude (167 m above sea level in the north, and 3327 m above sea level in the south) which influence the local climate and flora. In fawns (younger than 1 year) inhabiting the southern part of Belluno, average mandibular length was determined at 128.8 mm in 1990-1995 and 127.7 mm in 1996-2001. In the northern part of the examined region, the value of this parameter reached 123.6 mm and 123.9 mm, respectively. The average mandibular length in one-year-old males was determined at 153.3 mm in the south and 150.00 mm in the north. The analyzed parameter in 2-year-old bucks reached 157.4 mm in the south and 154.0 mm in the north, and in 2-year-old does – 156.1 mm in the south and 153.0 mm in the north. The above results indicate that the southern part of the Belluno region is characterized by a more supportive environment for roe deer. The observed variations in mandibular length were cross-referenced with the density of roe deer populations in the studied areas. Animal density was higher in the north (0.44 animals per km^2), and it decreased towards the south (0.33 animals per km^2). These results indicate that the density of animal populations also influences mandibular length (Zanneṡe et al., 2006b).

In the present study, the average mandibular length of fawns (up to 1 year of age) from the Czech Republic was determined at 129.2 mm, and was higher than that observed in Italian fawns. In older animals, the rate of mandibular growth was slower in Czech than in Italian roe deer. One- and 2-year-old individuals from the Czech Republic had shorter mandibles than Italian roe deer living in less favorable environmental conditions (145.7 mm and 151.1 mm on average, respectively). Our findings point to limited availability of food, high population density, a different genetic pool as well as differences in ecotype.

The results of our study and literature data can be used to develop a new strategy for monitoring the quality of local populations of European roe deer. Seasonal variations in bone size within one subpopulation are correlated mainly with environmental factors. Bone growth is most highly correlated with climate and population density. The body mass of fawns in winter, mandibular length in adult roe deer and foot length are most highly correlated with population density. In cervids, foot bones begin to grow rapidly immediately after birth, and their growth is completed relatively early, which is why this parameter is sensitive to environmental conditions. The length of hind feet varied across regions and was lower in areas characterized by lower availability of food, less supportive habitats and higher population density, regardless of gender (Zanneṡe et al., 2006a).

Conclusions

The results of this study indicate that mandibular length can be a reliable and easy to measure indicator of the quality of individual roe deer and, indirectly, roe deer subpopulations. The mandibular length and body mass of roe deer can also be robust bioindicators of habitat quality. Both parameters can be used in practice by wildlife specialists and practitioners responsible for managing free-living deer populations. Further, detailed research should be extended to include other species of game and protected mammals.

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SPATIO-TEMPORAL MOBILITY OF APICULTURE AFFECTED BY THE CLIMATE CHANGE IN THE BEEKEEPING OF THE GULF OF MEXICO

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Abstract. One the effects of climate change on populations has been the alteration in the geographical limits and spatial mobility of plants, which generates changes with associated species, such as honeybees which could produce changes in beekeeping. The aim of this study was to determine the spatio-temporal mobility of beekeeping, in the short, medium and long term, in the main honey-producing area in the Gulf of Mexico`s beekeeping region due to changes in temperature resulting from climate change using a regional assembly model with scenario A2. The productive potential of eight indicator melliferous species was determined: *C. sinensis, C. paradisi, B. simaruba, C. arabica, A. germinans, R. mangle, B. nigra* and *S. mombin*. The development potential for *A. mellifera* was determined based on combining bee thermal comfort and melliferous species' information, forming four categories for apicultural development. Based on the type of flowering, beekeeping will be confronted by a loss of suitable area for citrus species and an increase in area for the rest of the indicator species, which demonstrated different changes according to each species from the short to the long term. Therefore, beekeeping will have a varied spatio-temporal mobility according to the availability of botanical resources and the thermal comfort of the honeybee.
Keywords: *apiarian mobility, melliferous flora, beekeeping and climate change, Apis mellifera, honey*

Introduction

Global temperature increases forecasted by the Intergovernmental Panel on Climate Change are estimated to range from 1.4 to 3.1 °C over the next 100 years (IPCC, 2014). This will affect individuals, populations and species, as well as ecosystems, resulting in changes in the geographical limits and spatial and elevational mobility of plant communities. This will generate changes in space and time in ecosystem relationships with associated species (Bravo et al., 2011, Zhou et al., 2016).

Among the ecological relationships, the one which exists between plants and bees is one of the most valued since 35% of global food production comes from crops pollinated by bees (Saturni, 2016), and also because beekeeping is an economic activity seen as a tool for the development of the primary sector in different countries (Huerta, 2008).

In beekeeping, the honey yields that determine the sector's profitability are related to the weather conditions that directly and indirectly affect honeybee (*Apis mellifera* L.) dynamics (Delgado et al., 2012, Mendizabal, 2005) and plant phenology (Hegland et al.,

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2009). Therefore, phenological processes related to thermo-pluvial variations are one of the main factors influencing ecosystems (Hildrew et al., 2017), as a result of climate change (Gomez-Diaz et al., 2007). Such variations affect bloom times due to increases in temperature, generating imbalances with pollinators associated with the vegetation (Tam and McDaniels, 2013).

Variation in vegetation phenology is an indicator in climate change ecosystems (Zhou et al., 2016). At regional scales, this impact has been underestimated for honey production (Delgado et al., 2012), which depends on the comfort state provided by the balance between the physiology of an organism and the environment that surrounds it, for optimum food transformation (Ruiz et al., 2011).

Therefore, the presence of tropical storms, hurricanes with destructive force and droughts as a result of climate change have an impact on bee production (Guemes et al., 2003), as well as its profitability (Magaña et al., 2016). In Mexico, honey production has maintained a downward trend (SIAP, 2015), which is attributed to, among other factors, the presence of erratic storms, early frosts and droughts that are not conducive to optimum flowering development (Contreras-Escareño et al., 2013). In 2005 and 2007, a 11.1% reduction in honey production coincided with the presence of Hurricanes Wilma and Dean on the Yucatan Peninsula. However, in 2009 and 2010 a combination of the ENSO neutral and negative (La Niña) phases contributed to a 6.1% drop in domestic honey production (CONAGUA, 2011, SIAP, 2015).

Therefore, an alteration in the climatic system can define a new spatial distribution of bees intervening in the foraging relationships between species and races, as well as in the development of associated parasites and pathogens. This will force beekeepers to change their production methods in the coming decades (Le Conte and Navajas, 2008).

In countries such as Puerto Rico, the trend in terms of areas for honey production has been evaluated with climate projection models, based on future climate change scenarios. This is where a risk is foreseen in the form of a predicted reduction in the spatial areas suitable for apicultural development (Delgado et al., 2012).

The study of changes in beekeeping through future scenarios that allow projecting possible changes induced by climate change, based on the plant-insect relationship in tropical regions, would enable exploring the availability of suitable areas for apiculture in space and time, for the development of use and adaptation strategies.

In this regard, the aim of this research was to determine the spatio-temporal mobility of beekeeping in the central region of the state of Veracruz along the Gulf Coast in the short (2021-2030), medium (2041-2050) and long term (2051-2060), due to changes in temperature and precipitation resulting from climate change predicted by the SICC regional assembly projection model under scenario A2 conditions (IPCC, 2007). The information thus generated is intended to provide a comprehensive overview of the impacts that this phenomenon will have on beekeeping in this important apiarian region, considering the spatial area available for the development of melliferous species.

Materials and Methods

The study area was the main production area in the beekeeping region of the Gulf of Mexico (Villegas et al., 2003), located between 97° 27' 0'' and 95° 26' 41.9'' WL and 18°31' WL. This region comprises 20 municipalities in the state of Veracruz, namely: Altotonga, Alvarado, Atzalan, Boca del Rio, Coatepec, Comapa, Córdoba, Cosautlán de

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The indicator melliferous species that account for the bulk of honey production and that have a high weighting in terms of the number of individuals per hectare were identified. This information was based on the records of the transhumance routes in the region (PRONATURA-Veracruz, 2010), as well as information provided by n= 88 beekeepers on the main blooms on each of the routes.

It was taken as a sample beekeepers population who issued record levels of infestation by *Varroa destructor* Anderson and Trueman (2000), as issued by the national governing body in the state delegation of SAGARPA. To estimate the sample size from a population of N=247, a simple random sampling technique was used (Scheaffer et al., 1987), where:

$$n = \frac{N\sigma^2}{(N-1)D + \sigma^2}$$

N = Number of hives
n = Sample size
σ = Standard deviation
D = Error disposition

Sample size (n = 88) was obtained by replacing the following values in the formula: N = 247, the standard deviation of the number of hives (σ) = 200.6 and B = 34.4, with α = 0.95.

We used the Mexican productive crop potential method (Diaz et al., 2012) and geographic information system tools (scale 1:50,000), along with Series III climate (precipitation and temperature) data from the National Institute of Statistics and Geography (INEGI) and edaphoclimatic requirements of indicator species such as temperature, precipitation, elevation, soil type, depth and texture, and the salinity variable for the species that required it. Vector images were developed at the INIFAP Teocelo Experimental Site's Digital Agromap Laboratory; the images were reclassified at two levels: the high (1) and medium (2) development area based on the edaphoclimatic requirements of the species (Ruiz et al., 2011).

In parallel, the response in *A. mellifera* L. development to the environmental temperature was defined to predict the potential geographical distribution amplitude of the species, using the cartographic process employed for the productive potential of the melliferous plants.

In the final stage, an intersect of the resulting vector models between plants and bees was made to determine the spatial distribution for the beekeeping activity in the desired time period. The INIFAP National Environmental Information System (SIA for its acronym in Spanish) was used, with historical normal values for the period 1961-2003 (Ruiz et al., 2003), which was worked in raster format, adding algebraically the estimated future anomalies by Magaña and Caetano (2007) in their model SICC. The simulation of climate changes due to climate change in the short (2021-2030), medium (2041-2050) and long term (2051-2060) was based on the SICC assembly model proposed by Magaña and Caetano (2007) with weighted climate values from 10 General Circulation Models (mpi_echam5, miub_echo_g, csiro_mk3_0, csiro_mk3_5, cccma_cgcm3_1, giss_model_e_r, ncar_ccsm3_0, miroc3_2_hires, mri_cgcm2_3_2a,

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ukmo_hadcm3), with a resolution of 90 m x 90 m. This assembly presented changes according to scenario A2 contained in the AR4 published by the IPCC (2007).

With the SICC and SIA we calculated deviations of the study variables between the 1961-2003 climatology and the decades of short (2021-2030), medium (2041-2050) and long term (2051-2060) showing that the temperature will have an increase of 1.8 to 2 °C, from tropical regions and high valleys such as the apicultural region of the study área.

The indicator melliferous species used for the present study were: *Citrus sinensis* (L.) Osbeck (lemon), *Citrus paradisi* Macf. (grapefruit), *Bursera simaruba* (L.) Sarg. (gumbo-limbo), *Coffe arabica* L. (coffee), *Avicennia germinans* L. (black mangrove), *Rizophora mangle* L. (red mangrove), *Brassica nigra* (L.) Koch (black mustard) and *Spondias mombin* L. (jobo) (*Figure 1*).

In the mapping process, waterbodies and urban areas were excluded because they included wild plants that did not correspond to specific crop areas. In addition, due to the similarity in edaphoclimatic requirements, the two citrus (*C. sinensis* and *C. paradisi*) and mangrove forest (*A. germinans* and *R. mangle*) species were grouped into two flowerings: citruses and mangrove. The changes in the area with productive potential for melliferous species were presented in units of hectares to quantify the changes in the spatial and temporal dimension based on the projection model.

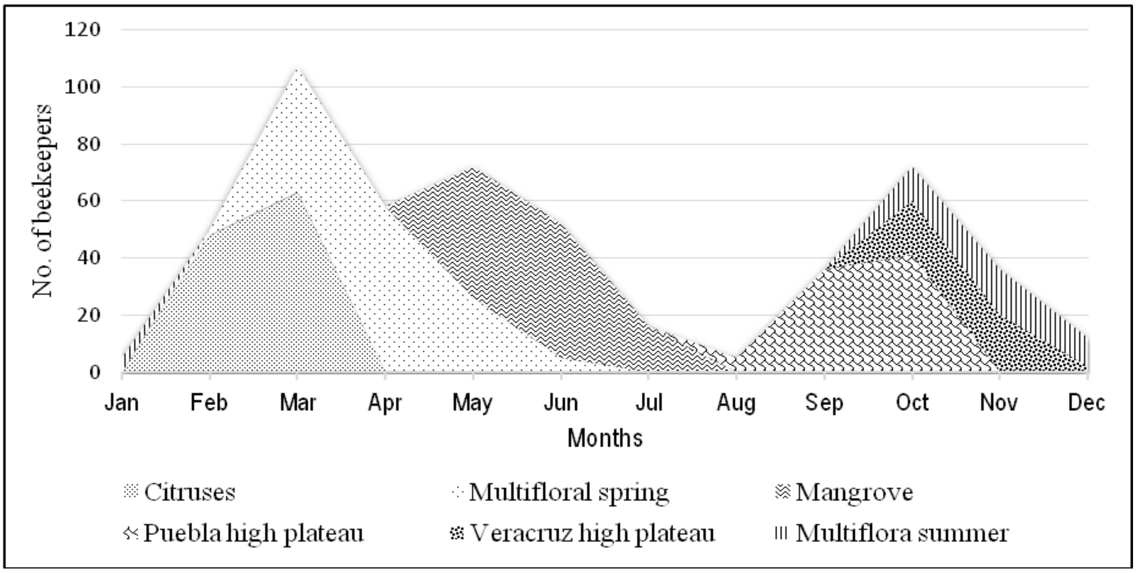

Figure 1. *Temporality of blooms available in the beekeeping region of the Gulf of Mexico, based on the percentage of beekeepers*

Results and Discussion

Mobility of the melliferous flora

Based on the projection model, it was found that the cultivated citrus species (*C. sinensis* and *C. paradisi*) showed a reduced area with high potential in the short (57.6%), medium (70.1%) and long term (75.2%). The replacement of the area with high potential, by areas with characteristics of medium potential, reflected that the predictions of the simulation model for these species are negative. However, by being

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cultivated species, the impacts could be cushioned by human actions rather than by any response mechanisms they may have (*Table 1*).

Table 1. *Percentage change in the melliferous flora area at temporal scale*

		Level of change			
Period	**Indicator species**	**High**	**Trend**	**Medium**	**Trend**
Short term	Citruses	-57.6	↓	56.6	↑
(2021-2030)	*B. simaruba*	4.7	↑	-8.2	↓
	C. arabica	2.1	↑	8.3	↑
	Mangrove	0.0	=	0.0	
	B. nigra	-4.1	↓	0.9	↑
	S. mombin	12.0	↑	-12.4	↓
Medium term	Citruses	-70.6	↓	69.4	↑
(2041-2050)	*B. simaruba*	5.1	↑	-7.9	↓
	C. arabica		↑	-4.1	↓
	Mangrove	0.0	=	0.0	
	B. nigra	-3.3	↓	0.7	↑
	S. mombin	13.6	↑	-14.1	↓
Long term	Citruses	-75.2	↓	73.9	↑
(2051-2060	*B. simaruba*	5.0	↑	-8.0	↓
	C. arabica	-1.9	↓	-11.6	↓
	Mangrove	0.0	=	0.0	
	B. nigra	-3.0	↓	0.6	↑
	S. mombin	4.0	↑	-4.2	↓

The sign (-) indicates reduction in area percentage value.

The citrus species involved in transhumant beekeeping are cash crops where production depends on characteristics such as technical handling, input and management, technological efficiency, soil, pests and diseases, productivity per tree and per hectare, management requirements, shipping costs and logistics (Neves et al., 2012).

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Thus, impacts caused by climatic variations that jeopardize production are only determined by the geographical and temporal location of these crops (Rosenzweig et al., 1996), so that climate change will exacerbate their spatial distribution problems (Fitchett et al., 2014).

For its part, *C. arabica* (coffee) is another cultivated indicator melliferous species that improves its production yields due to the presence of bees (Saturni et al., 2016). In this regard it was observed that in the short and medium term, the development area with high potential showed an increase of only 2.1 and 2.0%, respectively, compared to the current potential area, but in the long term it had a reduction of 1.9% with respect to the current area values. Therefore, the variation between one period and another did not allow demarcating a positive or negative spatial trend (*Table 1*).

Although *C. arabica* showed a lower reduction percentage in the area with high productive potential, it coincides with reports from the Southern Sudan in Africa, where the loss of optimum area for coffee cultivation, under the A2A scenario, is up to 68%. However, in contrast to the results of the model used in this study, the area of intermediate potential is reduced by 11.6%, whereas in Africa this area increases by 95% until the 2080s (Davis et al., 2012).

While the change in projected areas is greater in that reported by Davis et al. (2012), the reduction in the area with optimum conditions for coffee cultivation is confirmed in this study and the percentage difference between the two can be attributed to geographic factors and the structure and approach of the type of climatic modeling used (Zhang et al., 2015).

For the species *B. nigra*, the projection model showed a negative impact for the area with high development potential, with a 3% reduction in relation to the current area from the short to the long term (*Table 1*).

For their part, the mangrove species (*A. germinans* and *R. mangle*) showed no spatial changes in areas with high productive potential, so based on the A2 scenario these plant communities will not undergo changes (*Table 1*). However, the IPCC predicts that coastal systems will experience adverse impacts from inundation, flooding and coastal erosion, which can lead to a loss of area suitable for the development of these species (IPCC, 2014). Because they are bio-complex systems that involve changes in the relative increase in sea level, temperature and soil chemical conditions, mangrove ecosystems require a comprehensive approach to ecosystem management to understand their operation and the effects that climate change entails (Yañez-Arancibia et al., 2014, Day et al., 2008).

One hypothesis is that these ecosystems could benefit by undergoing a spatial and temporal expansion due to the effects of climate change, based on a possible tropicalization of the Gulf of Mexico (Yañez-Arancibia et al., 2014) excluding the impacts mentioned, as well as those generated by demographic, economic and urban growth that put the structure, composition, function and distribution of these ecosystems at risk (IPCC, 2014, Yañez-Arancibia et al., 2014).

The species that expanded their development areas were those that make up part of the wild vegetation, *B. simaruba* and *S. mombin*, as part of the spring and autumn flowering. In both species the climate projection model predicts a positive impact in the short, medium and long term, increasing the development area with high potential by 5% for *B. simaruba* and 4% for *S. mombin*, with respect to the current area.

The amplitude of intervals in the edaphoclimatic development characteristics of the species gave them greater tolerance to the changes that the model projects, which is why

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an increase in the development area in comparison with the cultivated species is predicted. However, according to the fifth IPCC report (2014), tree mortality and forest decline due to rising temperatures will increase in different regions, jeopardizing carbon storage, biodiversity, timber production, water quality and aesthetic value.

Therefore, whether there is an increase or reduction in the development area of the indicator species as a consequence of global warming will be subject to the dispersion ability of each species, based on its edaphoclimatic characteristics, tolerance intervals and associated fauna. As a result, spatial mobility will be different for each type of species or set of plant populations (Yañez-Arancibia et al., 2014).

Thermal comfort of A. mellifera

It was found that the space projected with optimum comfort conditions for bee (*A. mellifera*) development increased. Based on the thermal factor, the high potential development area showed an expansion in spatial distribution of 19% in the short term, 23.40% in the medium term and 25.4% in the long term, while the medium potential area decreased by 32.6% in the short term, 40.9% in the medium term and 45% in the long term, which indicates an increase in the geographical distribution, as a consequence of the variations in temperature that climate change entails in the A2 scenario**.**

These results confirm that temperature is one of the main determinants of the ecological role played by species (Hildrew et al., 2017) and biogeographical distribution (Jeffree and Jeffree, 1994, Bravo et al., 2011). Therefore, the amplitude of the geographical space for the bee (*A. mellifera*) predicts that it will favor the expansion in the current limits of areas with thermal comfort. As has been reported for other insects, bees will have a clear spatial mobility (Regniere, 2009; Kiritani, 2013). However, landscape modification and extensive agriculture are constant risk factors that promote the fragmentation of suitable habitats for beekeeping (Burkle et al., 2013) (*Table 2*).

Table 2. *Percentage change in thermal comfort area for A. mellifera from short to long term*

Potential/period	Short	Trend	Medium	Trend	Long	Trend
High	19.0	↑	23.4	↑	25.4	↑
Medium	-32.6	↓	-40.9	↓	-45.0	↓

The sign (-) indicates reduction in area percentage value.

Spatio-temporal mobility of beekeeping areas

With the intersects, the area with optimum thermal comfort for bees and optimum development potential for melliferous plants in each type of flowering was projected.

In the citrus species (*C. sinesis* and *C. paradisi*), negative changes were observed due to a 57.6% loss of spatial distribution in the optimum area for beekeeping development from the short (2021-2030) to the long term (2051-2060), as well as in the species *S. mombin*, which showed a 12% reduction in distribution for the same period (*Table 3*) (*Figure 2*).

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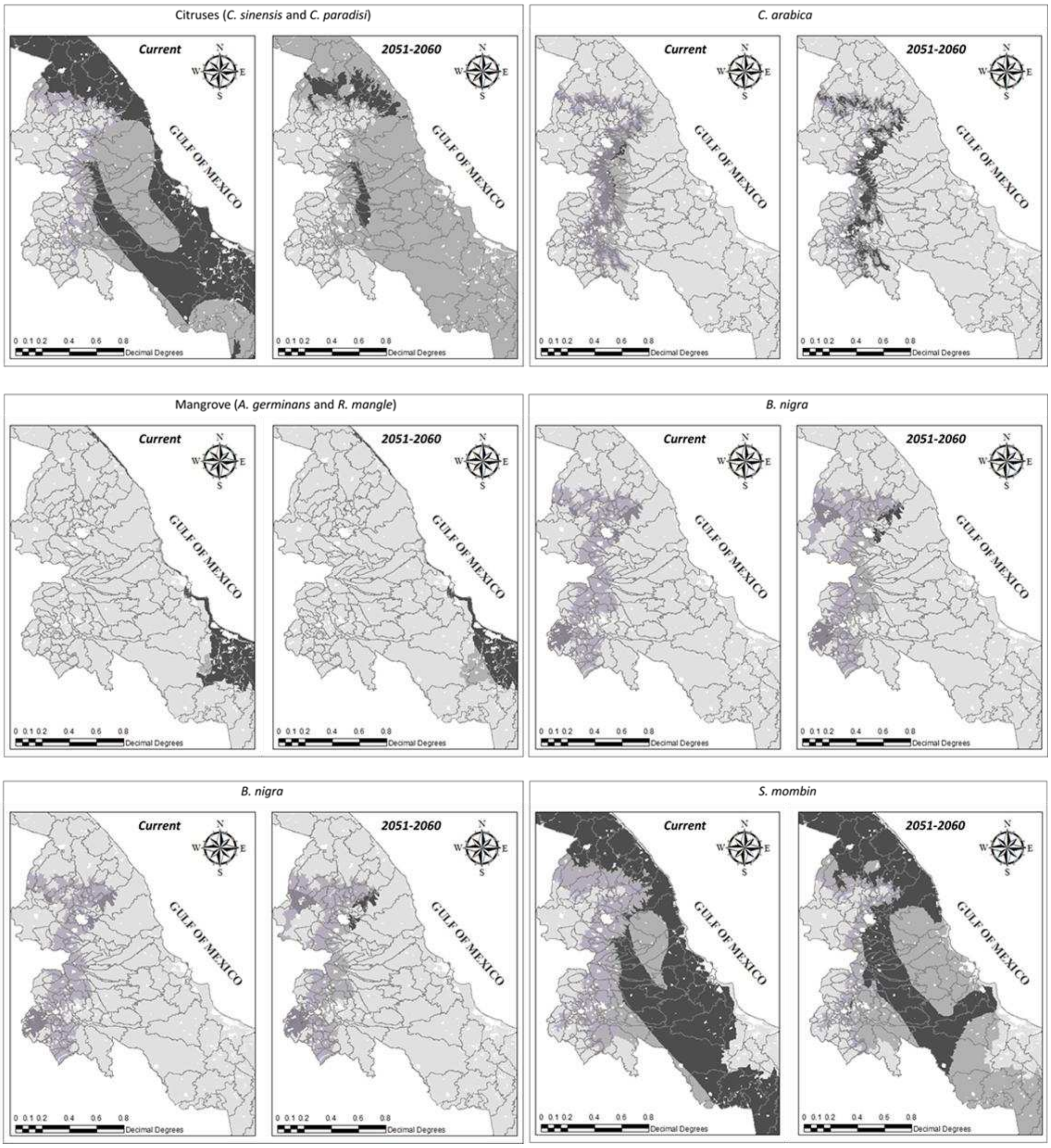

Figure 2. *Predicted impact of climate change on beekeeping under current and future climatic conditions (scenario A2, IPCC)*

Table 3. *Percentage of the area of change in crosses made*

Period	Indicator spp	Plant(+) Bee (+)		Plant(+) Bee (-)		Plant(-) Bee (+)		Plant(-) Bee (-)	
Short term	Citruses	-57.6	↓	--------	--	137.0	↑	-81.0	↓
	B. simaruba	12.3	↑	-100.0	↓	16.0	↑	-57.6	↓
	C. arabica	683.2	↑	-70.9	↓	37.6	↑	-16.1	↓
	Mangrove	1.0	=	--------	--	--------	--	--------	--
	B. nigra	9266.0	↑	41.4	↑	18514.0	↑	-16.9	↓
	S. mombin	-12.0	↓	--------	--	51.0	↑	-52.0	↓

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Medium term	Citruses	-70.6	↓	--------	--	163.5	↑	-91.9	↓
	B. simaruba	13.0	↑	-99.6	↓	21.9	↑	-68.1	↓
	C. arabica	835.3	↑	-87.3	↓	29.3	↑	-31.9	↓
	Mangrove	1.0	=	--------	--	--------	--	--------	--
	B. nigra	9332.9	↑	42.5	↑	28946.0	↑	-29.7	↓
	S. mombin	14.0	↑	--------	--	66.0	↑	-65.0	↓
Long term	Citruses	-75.2	↓	--------	--	-172.8	↓	-95.7	↓
	B. simaruba	13.0	↑	-99.4	↓	-24.2	↓	-72.6	↓
	C. arabica	850.2	↑	-93.3	↓	24.3	↑	-41.4	↓
	Mangrove	-2.0	↓	--------	--	--------	--	--------	--
	B. nigra	9430.1	↑	42.5	↑	35056.0	↑	-37.1	↓
	S. mombin	4.0	↑	--------	--	101.0	↑	-70.0	↓

By contrast, the projections for the species that are expected to broaden their spatial distribution due to an increase in the optimum development area for beekeeping from the short to the long term were the association of the bee with *B. simaruba*, *B. nigra*, and *C. arabica*, showing an expansion in area of the region suitable for bee development (*Table 3*) (*Figure 2*).

For their part, mangrove species (*A. germinans* and *R. mangle*) showed no changes in development area, maintaining the current area in the apicultural zones of the region in the three projected time periods. Therefore, the model does not foresee significant changes in beekeeping in these ecosystems due to the climatic variants (*Figure 2*).

Beekeeping is an activity that is established on the basis of available flowering, which is considered as one of the main elements in the industry (Khabbach et al., 2013, Al-Ghamdi et al., 2016). In this research it was observed that the distribution of apicultural zones with high potential for the insect and melliferous plants was not defined by any specific characteristic. However, the negative trend in citrus areas entailed by an average 1.9 °C temperature increase up to the long term (2051-2060) is a result of the effect of climate change.

This confirms what has been found in other studies (Delgado et al., 2012, Davis et al., 2012, Le Conte and Navajas, 2008, Redi et al., 2012, Lehébel-Péron et al., 2016) where it has been stated that increases in temperature, precipitation and relative humidity will affect the areas suitable for apicultural development, from phenological time-shifts to landscape modification, influencing the volume of production.

Because *A. mellifera* is generalist, it is believed that it may modify its foraging behavior in relation to the type of vegetation present (Gaines-Day and Gratton, 2016), since the intensity of visits is varied among the heterogenous blooms of the natural habitat, compared to crop blooms (Saturni et al., 2016).

The results of reclassifying the sites allowed confirming that there may be geographical expansion in areas with thermal comfort conditions for bees, congruent with the antecedents that foresee the territorial expansion of insect populations mainly due to the effect of temperature (Musolin and Saulich, 2012, Regniere, 2009, Le Conte and Navajas, 2008, Kiritani, 2013). Therefore, the thermal variations predicted in the A2 climatic scenario, of 1.9 °C up to the 2051-2060 decade in the area (Ruiz et al., 2011), could represent an advantage for the development of bees in the beekeeping

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region of the Gulf of Mexico and contribute to a greater number of areas that offer comfortable thermal development conditions for the bee.

It is thus expected that the transhumance routes, used by 91% of the beekeepers (Castellanos-Potenciano et al., 2015) in this apicultural region, will have a spatial and temporal mobility as a consequence of the changes in potential development areas, since climate fluctuations influence the availability of botanical resources (Medina-Cuéllar et al., 2014); however, the background of landscape modification, extensive agriculture and fragmented and deteriorated ecosystems where beekeeping is currently established (Burkle et al., 2013, Lehébel-Péron et al., 2016, Redi et al., 2012) continue to put this activity at risk.

Conclusion

In the short (2021-2030) medium (2041-2050) and long term (2051-2060), the indicator melliferous flora of the center of the state of Veracruz along the Gulf of Mexico will have a different spatio-temporal mobility, due to changes in temperature and precipitation as a result of climate change.

On the other hand, the current thermal comfort zone of *A. mellifera* L. in the region will have an expanded territory and the spatio-temporal mobility will be positive in the short, medium and long term as a result of temperature change due to the effect of climate change, which will manifest itself in optimum honeybee development.

Based on the intersects, it was concluded that apiculture will have a varied spatio-temporal mobility in the short, medium and long term according to the availability of botanical resources and the thermal comfort of the bees, since according to the characteristics of each species, there will be a reduced territory for citrus species, linked to a negative impact on the high potential development area. On the other hand, the other six species will have an expanded territory that will result in a positive impact for the apicultural development of the region, as a function of the climatic characteristics.

Under the future scenario of beekeeping in this area, there will be an increase in the optimum area for honey production, due to the effect of climate change (temperature and precipitation) on spatio-temporal scales. It is therefore recommended that future research include new IPCC climate projection scenarios, the variation in floral phenology, the genetic adaptation potential of *A. mellifera* races and historical honey production volumes, as well as consider other climatic variables such as radiation and the chemical composition of the atmosphere.

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FEW DOMINANT NATIVE WOODY SPECIES: HOW SUBTROPICAL RAINFOREST SUCCESSIONAL PROCESS ACTS ON ABANDONED PASTURES IN SOUTHERN BRAZIL

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Abstract. In Brazil, a high number of woody species have been used for forest restoration plantings. However, this action is widely questionable due to the no re-establishment of a considered “normal” successional trajectory. In this study, we analyzed subtropical rainforest natural regeneration at abandoned pastures and we showed that vegetation is characterized, predominantly, by few high dominant native woody species, highlighting especially *Vernonanthura discolor* (Asteraceae), *Myrsine coriacea* (Primulaceae), and *Piptocarpha regnellii* (Asteraceae). Our results also indicate that these high dominance species favor the lowest diversity of natural regeneration. In this regard, we suggest implementing actions that provide improvements and facilitate natural processes of ecological succession by planting dominant native woody species. This takes into consideration the re-establishment of a considered “normal” successional trajectory.
Keywords: *forest regeneration; “normal” successional trajectory; dominant species; species diversity; CSR ecological strategies*

Introduction

The subtropical forests are exposed to a continuous degradation process, due to anthropic landscapes changes (Ribeiro et al., 2009; Vibrans et al., 2013a). These landscapes are mainly pastures mosaics, crops and urban areas fringed by small forest patches (Tabarelli et al., 2010). The forests conversion into cattle raising pastures is known for reducing biological diversity, interrupting the ecological processes (Tinoco-Ojanguren et al., 2013), and also reducing water infiltration into the soil due to soil compaction by animals trampling, what increases superficial runoff and soil erosion (Kunz et al., 2013). In these areas, natural regeneration of native species is limited by a variety of processes coming from the conversion forest–pasture, including land degradation (Holl and Aide, 2011), unfavorable microclimate (Pröll et al., 2015), lower seed dispersion (Reid et al., 2015) and competition from invasive exotic species (Mantoani and Torezan, 2016).

There are ~177.282,00 km² of potential areas for forest restoration in Brazil (Rodrigues et al., 2011). These areas are generally situated in highly fragmented forest regions and present low agricultural potential due to massive livestock farming (Rodrigues et al., 2009). Such as in other Brazilian regions, Santa Catarina Atlantic

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Forest is composed of secondary forest physiognomy with different regeneration stages, being rare the remaining with primary forests (Reis et al., 1992). The whole area has suffered destructive extractivism and disorderly soil occupation for agriculture and livestock expansion (Vibrans et al., 2013b). Although, secondary forests are important for global biological diversity conservation (Gibson et al., 2011), in Southern Brazil there are few studies on species diversity of natural regeneration at the secondary succession process of subtropical forests (Meyer et al., 2013; Fiorentin et al., 2015).

If we understand the secondary succession process of abandoned pastures and priority areas indicated for restoration in Brazil (see Rodrigues et al., 2011; Brasil, 2012; 2017), we can show potential species to be used in the forest regeneration projects (Martins, 2013; Maçaneiro et al., 2016a; Mota et al., 2017; Turchetto et al., 2017). For example, species that colonize abandoned pastures are typical from disturbed environments, once they frequently occur in open areas (such as clearings) or, on the edge of forests, where environmental conditions are unfavorable for most of the demanding plants (Chazdon and Guariguata, 2016). Besides this, those species are adapted to local environmental conditions, characterizing native regeneration vegetation in the initial stages and, therefore, being recommended for use on subtropical forests restoration (Kageyama and Reis, 1993; Meli et al., 2014; Mota et al., 2017).

Heliophytic and light demanding plants are among the species prepared to take place at abandoned pastures (Chazdon, 2008; Cheung et al., 2009). They are highly adapted to unfavorable microclimate conditions (higher light levels) and degraded soil (compacted and low in nutrients) (Holl and Aide, 2011). These species are also often described as single-dominants or monodominants (see Connell and Lowman, 1989; Hart et al., 1989), since they occur in large numbers, have relative density or relative dominance between 50-100%, and dominate the forest canopy. Some studies were developed in Santa Catarina with the purpose to understand both the natural regeneration composition and structure of the Atlantic Forest (see Schorn and Galvão, 2009; Siminski, 2009; Meyer et al., 2013; Fiorentin et al., 2015; Higuchi et al., 2015; Maçaneiro et al., 2016a). Those studies verified that successional trajectories vary in function of the land use and the anthropic history. Although its descriptive content focus, those researches contribute to meta-analysis studies and also serves as basis for forest restoration projects in similar areas (Mota et al., 2017; Turchetto et al., 2017). However, none of these studies emphasized the relationship between natural regeneration dominant species and diversity in abandoned pastures.

The use of a large number of woody species in plantations for forest regeneration purpose is a widely questionable action, although it is a traditional practice in Brazil (Naeem, 2006; Wright et al., 2009; Durigan et al., 2010; Durigan and Engel, 2015). For instance, a degraded ecosystem is a highly organizaded system opened to matter and energy flows, with dissipative structure, presenting internal (among the system components) and external interactions (with the landscape) (Aumond and Maçaneiro, 2014). In this context, the answer for how many species would be necessary in order to have a stable community and a functional ecosystem must take into account how a considered "normal" successional trajectory re-establishment happens (see Suding and Gross, 2006; Naeem, 2006; Durigan and Engel, 2015). Furthermore, there are few woody species that seems to dominate at the beginning of forest succession of the subtropical forests (see Klein, 1980; Schorn and Galvão, 2009; Siminski, 2009; Meyer et al., 2013). In this regard, the aim of this study was to analyze natural regeneration woody species composition, structure, diversity, and abundance at abandoned pastures

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in order to respond tree key issues: (1) How many and which are the species growing at a four-year abandoned pasture? The pasture mentioned was used by cattle raising for more than a half century. (2) Do distribution abundance patterns of the natural regeneration woody species present important implications to the choice of new species for forest restoration projects?

Material and Methods

Study area

The study area is inserted in Faxinal do Bepe locality, Serra do Itajaí National Park, state of Santa Catarina, Southern Brazil. The area is within the limits of Itajaí river watershed, being the river Warnow a sub-watershed. Faxinal do Bepe has a total area of ~250 ha, altitude which varies of 700 to 1,039 m s.n.m. and is located between 27°05' - 27°07' S e 49°11' - 49º13' W (*Figure 1*).

The climate is Cfa - humid subtropical climate, without dry season and with hot summer (Alvares et al., 2014). The average annual temperature range between 16-18 °C, with temperature average monthly varying between 12-14 °C in the coldest month (July) and 20-23 ºC in the warmer months (January and February). The annual relative humidity varies between 82-84% and the total annual rainfall is between 1,500-1,700 mm well distributed during the year (Pandolfo et al., 2002).

The predominant vegetation is Subtropical Upper Hills Broadleaved Evergreen Rainforest (*sensu* Oliveira-Filho, 2015), hereafter referred to as Subtropical Rainforest, inserted at Atlantic Forest Domain. Regarding the natural resources historical use, after the year 1953, it has initiated the colonization and occupation process at Faxinal do Bepe, that lasted until 2004. At that period, large part of the forests were submitted to selective logging and posterior conversion to vast pasture areas that, currently, are found abandoned and at an initial regeneration stage.

Data collection

We selected three four-year abandoned pastures which were at an initial regeneration stage (*Figures 1 and 2*). Each area was constituted by a slope with the same historical use. We used plots arranged in transects (Soares et al., 2012), to represent the possible greatest variation throughout the three areas with abandoned pastures. At each area we distributed, systematically, 15 sample plots of 10 x 20 m (200 m²), corresponding to 3,000 m² sampling area and making up 20% of the total studied. We disposed these sample plots in three transects, all of them starting at the base of the slope and ending at the top of the slope. We distanced these sample plots approximately 25 m each other and 35 m from the transects. At each sample plot we sampled the upper layer, characterized by live individuals with diameter at breast height (DBH) ≥ 5 cm. Inside each sample plot we inserted a 10 x 10 m (100 m²) subplot, to sample lower layer, characterized by individuals with height ≥ 50 cm and DBH < 5 cm.

We identified botanical material collected by comparison with exsiccates deposited at the Dr. Roberto Miguel Klein Herbarium of Fundação Universidade Regional de Blumenau (FURB) and, also, through taxonomic literature and FURB experts consultation. We used the species classification system proposed by APG IV (2016) and PPG I (2016).

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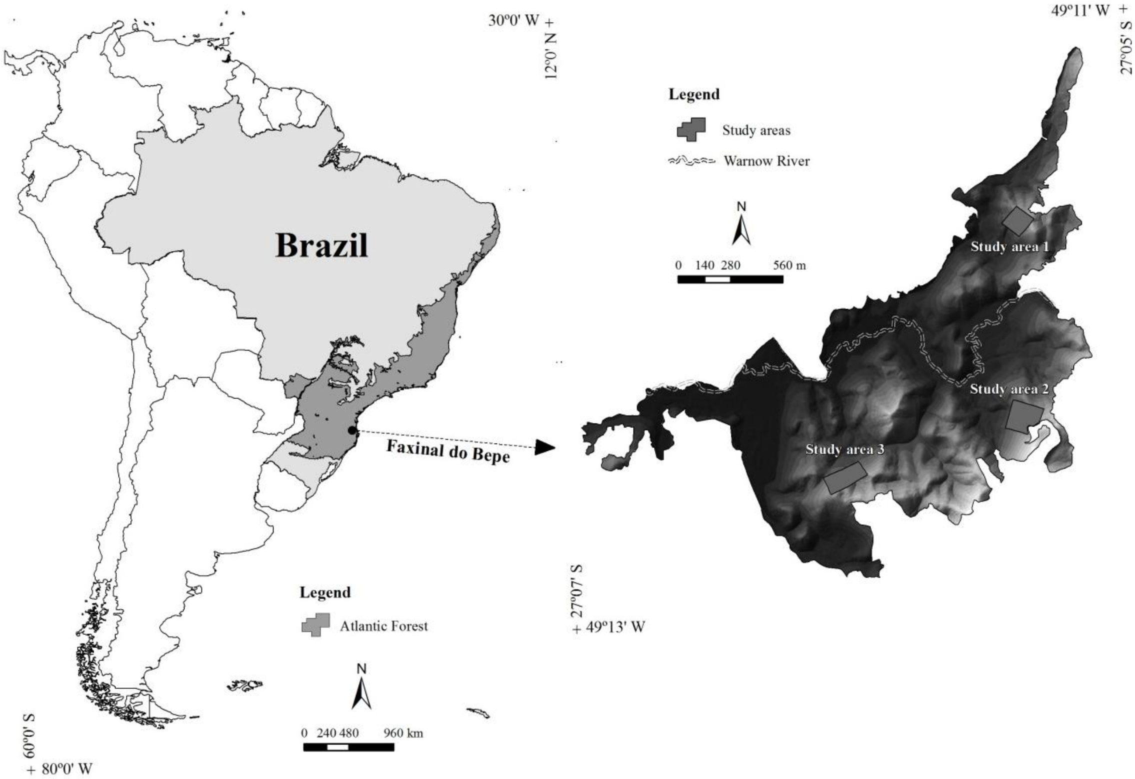

***Figure 1**. Studied area at Faxinal do Bepe, Serra do Itajaí National Park, Santa Catarina State, Southern Brazil.*

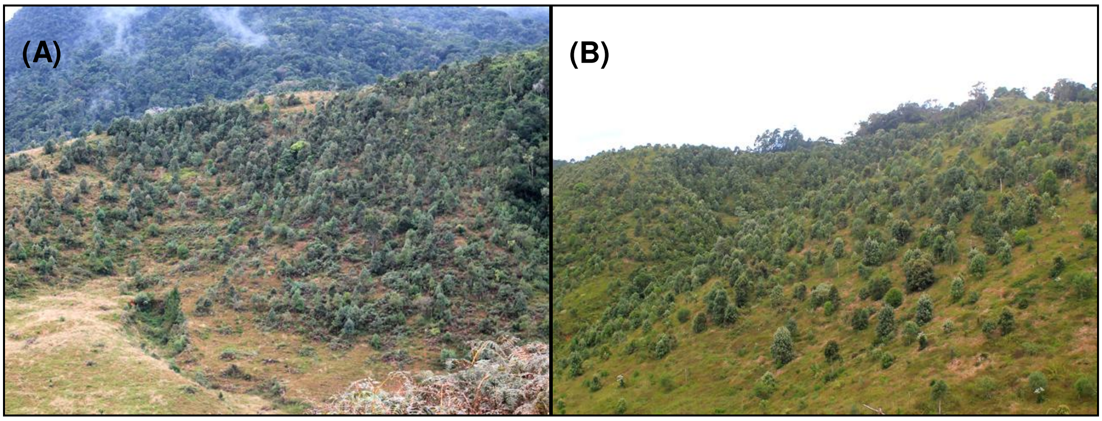

***Figure 2**. Photos of studied areas 1 (A) and 3 (B) at Faxinal do Bepe, Serra do Itajaí National Park, Santa Catarina State, Southern Brazil.*

Data analysis

We calculated, for upper layer, Mueller-Dombois and Ellenberg (2002) structural parameters, in other words, density, dominance and frequency absolute and relative, and importance value for each species. For lower layer, besides the parameter described above, we calculated absolute and relative height classes and the natural regeneration importance value for each species (Hosokawa et al.,

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2008). Afterward, we classified these species by CRS ecological strategy (see Grime et al., 1997), adopting the methodology suggested by Pierce et al. (2013) into the following categories: C – competitor specie with high potential growth rate and rapidly biomass expanding; S – stress-tolerant specie and slow-growing; R – ruderal specie with premature reproduction for prolonged period.

We verified species abundance distribution patterns (PDSA) of the natural regeneration by Whittaker diagram (Magurran, 2004). Similarities or differences between PDSA layers analyzed were verified by Kolmogorov-Smirnov test for two sample plots, at the significance level $\alpha = 0.01$ (Sokal and Rohlf, 2011). The Whittaker diagram is considered a useful tool to analyze species PSDA into plant communities, once contrasting patterns between species richness and vegetation uniformity can be clearly observed (Krebs, 2014; Maçaneiro et al., 2016b).

We estimated the vegetation heterogeneity (Krebs, 2014) by Shannon index (*H*', Napier's logarithms) and Simpson index (1-*D*). Afterwards, we converted these indexes to the effective number of species – ENS (see Jost, 2006) by the following expressions: Shannon index = exp(*H*') and Simpson index = 1/(1-(1-*D*)). Indexes *H*' and 1-*D* conversion into real diversity (effective number of species) giving it a set of common behaviors and properties, easily interpretable. After this conversion, the diversity is always measured as species number, regardless of the index used (Jost, 2010). Additionally, we used Pearson correlation coefficient and scatterplots to verify the relationship between the diversity and abundance of dominant woody species at the layers. First, we correlated relative density (*DR%*) of the specie with the greatest individual number of each sample plot with its respective *H*' e 1-*D* converted into ENS. Next, we investigated the statistical significance ($\alpha = 0.01$) of the correlations through *t* test for correlation existence (Zar, 2010). Finally, we constructed dispersion graphics between diversity evidences (axis y) and *DR%* (axis x), and inserted a linear trend line for the relation between *H*' and *DR%*, and 1-*D* and *DR%* , both converted into ENS.

Results

We sampled 1,079 individuals belonging to 45 woody species *(Table 1)* in both layers. The natural regeneration presented 497.8 ind.ha^{-1} and monodominance of *Vernonanthura discolor* (*DR* > 50%), in the upper layer. Besides *Vernonanthura discolor*, *Piptocarpha regnellii*, *Piptocarpha axillaris*, *Myrsine coriacea* and *Piptocarpha angustifolia* (*VI* = 266.2%) also characterize the upper layer.

In the lower layer, we found density of 1,402.2 ind.ha^{-1} and the mains species that characterized the vegetation structure were *Vernonanthura discolor*, *Myrsine coriacea*, *Clethra scabra*, *Piptocarpha regnellii* and *Piptocarpha axillaris* (*RNR* = 186.2%). Similar to what was found on the upper layer, *Vernonanthura discolor*, *Myrsine coriacea*, *Piptocarpha regnellii* and *Piptocarpha axillaris* also were the main species at the lower layer (*Table 1*).

We observed that woody species presented different ecological regeneration strategies (*Table 1*). However, competitors and stress tolerant plants (S/SC) were predominant at the analyzed layers (upper layer = 64.4%; lower layer = 70.5%).

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***Table 1**. Phytosociological parameters for woody species in two layers of natural regeneration of a Subtropical Rainforest in Southern Brazil.*

Upper layer								
Species	***DA***	***DR***	***FA***	***FR***	***DoA***	***DoR***	***VI***	**ES**
Vernonanthura discolor (Spreng.) H.Rob.	256.7	51.6	88.9	31.0	1.88	57.1	139.7	S/SC
Piptocarpha regnellii (Sch.Bip.) Cabrera	87.8	17.6	46.7	16.3	0.67	20.3	54.2	S/SC
Piptocarpha axillaris (Less.) Baker	58.9	11.8	42.2	14.7	0.25	7.5	34.1	S/SC
Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	31.1	6.3	33.3	11.6	0.13	3.8	21.7	S/SC
Piptocarpha angustifolia Dusén ex Malme	27.8	5.6	22.2	7.8	0.14	4.2	17.5	S/SC
Clethra scabra Pers.	10.0	2.0	15.6	5.4	0.03	0.8	8.2	S/SC
Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	6.7	1.3	11.1	3.9	0.05	1.4	6.6	R/CSR
Baccharis semiserrata DC.	7.8	1.6	8.9	3.1	0.06	1.9	6.5	S
Ocotea puberula (Rich.) Nees	3.3	0.7	6.7	2.3	0.01	0.3	3.3	SR/CSR
Annona emarginata (Schltdl.) H.Rainer	3.3	0.7	2.2	0.8	0.03	1.0	2.5	S/SC
Ocotea odorifera (Vell.) Rohwer	1.1	0.2	2.2	0.8	0.04	1.2	2.2	S/SC
Baccharis dracunculifolia DC.	1.1	0.2	2.2	0.8	0.01	0.2	1.2	S
Solanum lacerdae Dusén	1.1	0.2	2.2	0.8	0.004	0.1	1.1	S/SC
Aspidosperma tomentosum Mart.	1.1	0.2	2.2	0.8	0.003	0.1	1.1	S/SC
Total	497.8	100.0	286.7	100.0	3.30	100.0	300.0	-
Lower layer								
Species	***DA***	***DR***	***FA***	***FR***	***CAT***	***CRT***	***RNR***	**ES**
Vernonanthura discolor (Spreng.) H.Rob.	433.3	30.9	84.4	16.1	51.8	31.0	78.0	S/SC
Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	177.8	12.7	55.6	10.6	18.9	11.3	34.6	S/SC
Clethra scabra Pers.	126.7	9.0	51.1	9.7	15.4	9.2	28.0	S/SC
Piptocarpha regnellii (Sch.Bip.) Cabrera	115.6	8.2	48.9	9.3	13.9	8.3	25.9	S/SC
Piptocarpha axillaris (Less.) Baker	113.3	8.1	37.8	7.2	13.7	8.2	23.5	S/SC
Piptocarpha angustifolia Dusén ex Malme	57.8	4.1	28.9	5.5	7.0	4.2	13.8	S/SC
Miconia tristis Spring	53.3	3.8	20.0	3.8	6.0	3.6	11.2	S/SC
Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	33.3	2.4	20.0	3.8	4.4	2.7	8.8	R/CSR
Myrsine umbellata Mart.	35.6	2.5	20.0	3.8	4.0	2.4	8.7	S/SC
Solanum americanum Mill.	53.3	3.8	4.4	0.8	6.4	3.8	8.5	S/SC
Baccharis semiserrata DC.	31.1	2.2	13.3	2.5	2.7	1.6	6.3	S/SC
Miconia sellowiana Naudin	13.3	1.0	13.3	2.5	1.8	1.1	4.6	S/SC
Solanum mauritianum Scop.	13.3	1.0	13.3	2.5	1.7	1.0	4.5	S/SC
Ocotea puberula (Rich.) Nees	13.3	1.0	8.9	1.7	1.7	1.0	3.7	SR/CSR
Baccharis oblongifolia (Ruiz & Pav.) Pers.	8.9	0.6	6.7	1.3	1.2	0.7	2.6	S/SC
Baccharis dracunculifolia DC.	6.7	0.5	6.7	1.3	1.0	0.6	2.3	S/SC
Ficus luschnathiana (Miq.) Miq.	6.7	0.5	6.7	1.3	1.0	0.6	2.3	S/SC
Campomanesia guaviroba (DC.) Kiaersk.	8.9	0.6	4.4	0.8	1.4	0.8	2.3	S/SC
Solanum lacerdae Dusén	6.7	0.5	6.7	1.3	0.9	0.5	2.3	S/SC
Annona emarginata (Schltdl.) H.Rainer	6.7	0.5	6.7	1.3	0.9	0.5	2.3	S/SC
Inga vera subsp. *affinis* (DC.) T.D.Penn.	6.7	0.5	6.7	1.3	0.8	0.5	2.2	S/SC
Myrcia splendens (Sw.) DC.	8.9	0.6	4.4	0.8	1.0	0.6	2.1	S/SC
Syagrus romanzoffiana (Cham.) Glassman	6.7	0.5	4.4	0.8	0.9	0.5	1.8	S/SC
Miconia cabucu Hoehne	4.4	0.3	4.4	0.8	0.7	0.4	1.6	S/SC
Alchornea triplinervia (Spreng.) Müll.Arg.	4.4	0.3	4.4	0.8	0.5	0.3	1.5	S/SC
Ocotea elegans Mez	6.7	0.5	2.2	0.4	0.9	0.5	1.4	S/SC
Aspidosperma tomentosum Mart.	4.4	0.3	2.2	0.4	0.7	0.4	1.2	S/SC
Zanthoxylum rhoifolium Lam.	4.4	0.3	2.2	0.4	0.7	0.4	1.2	S/SC
Casearia sylvestris Sw.	4.4	0.3	2.2	0.4	0.7	0.4	1.2	SC/CSR
Cyathea phalerata Mart.	4.4	0.3	2.2	0.4	0.6	0.4	1.1	S
Guatteria australis A.St.-Hil.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	S/SC

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Critoniopsis quinqueflora (Less.) H.Rob.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	S/SC
Solanum variabile Mart.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	SC/CSR
Dalbergia brasiliensis Vogel	2.2	0.2	2.2	0.4	0.3	0.2	0.8	SC/CSR
Leandra carassana (DC.) Cogn.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	S/SC
Miconia inconspicua Miq.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	SC/CSR
Miconia lymanii Wurdack	2.2	0.2	2.2	0.4	0.3	0.2	0.8	S/SC
Cedrela fissilis Vell.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	R/CR
Rubus brasiliensis Mart.	2.2	0.2	2.2	0.4	0.3	0.2	0.8	S/SC
Handroanthus chrysotrichus (Mart. ex DC.) Mattos	2.2	0.2	2.2	0.4	0.3	0.2	0.7	SR/CSR
Jacaranda puberula Cham.	2.2	0.2	2.2	0.4	0.3	0.2	0.7	S/SC
Nectandra oppositifolia Nees	2.2	0.2	2.2	0.4	0.3	0.2	0.7	S/SC
Leandra glazioviana Cogn.	2.2	0.2	2.2	0.4	0.3	0.2	0.7	S/SC
Campomanesia reitziana D.Legrand	2.2	0.2	2.2	0.4	0.3	0.2	0.7	S/SC
Total	1,402.2	100.0	524.4	100.0	167.3	100.0	300.0	-

Note: DA: absolute density (ind.ha^{-1}); DR: relative density (%); FA: absolute frequency (%); FR: relative frequency (%); DoA: absolute dominance (m².ha^{-1}); DoR: relative dominance (%); VI: importance value (%); CAT: absolute size class; CRT: relative size class (%); RNR: relative natural regeneration (%); ES: ecological strategies: competitor (C), stress tolerant (S), ruderal adapted to disorders (R), ruderal competitor in environment subjected to stress and disorders (CR/CSR), ruderal competitor (R/CR), ruderal in environment subjected to stress and disorders (R/CSR), stress tolerant and competitor (S/SC), stress tolerant and competitor in environment subjected to stress and disorders (SC/CSR) and ruderal and stress tolerant in environment subjected to stress and disorders (SR/CSR).

Differences between vegetation layers are particularly visible at the community structure (*Figure 3*). We verified PDSA significantly different throughout the analyzed layers (Kolmogorov-Smirnov, D = 0.68; p < 0.01). However, Whittaker diagram showed that natural regeneration is characterized by few high dominant woody species, highlighting *Vernonanthura discolor*, *Myrsine coriacea* and *Piptocarpha regnellii*.

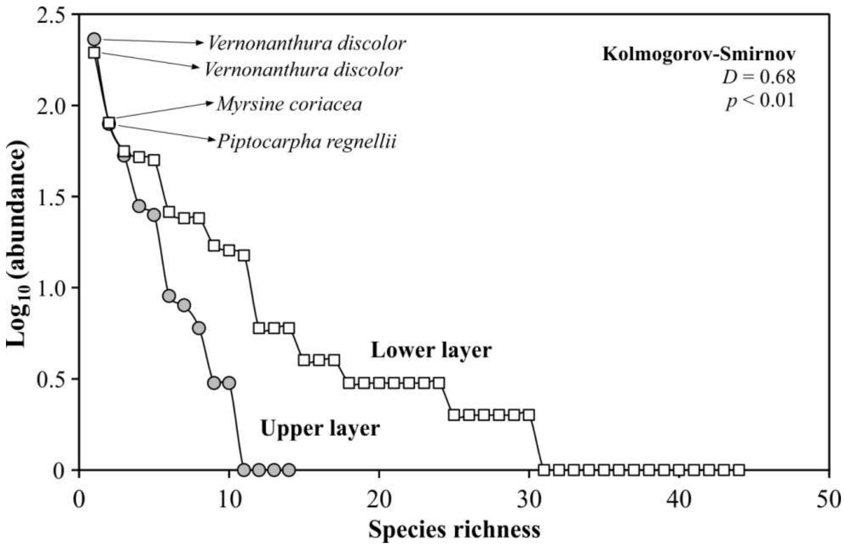

***Figure 3**. Whittaker diagram for two layers of natural regeneration of a Subtropical Rainforest in Southern Brazil.*

We found highly significant correlations (Pearson, r > -0.79; p < 0.01) between diversity index (Shannon and Simpson) and relative density of the mostly abundant woody species at the sample plots analyzed (*Table 2, Figure 4*).

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***Table 2**. Pearson coefficient correlation (r) between diversity indexes and relative density of the most abundant woody specie at the sample plots in two layers of natural regeneration of a Subtropical Rainforest in Southern Brazil.*

Diversity index	ENS	R^2	r	p
Upper layer				
Shannon	4.78	0.62	-0.79	<0.0001
Simpson	3.16	0.79	-0.89	<0.0001
Lower layer				
Shannon	13.00	0.68	-0.82	<0.0001
Simpson	7.21	0.78	-0.88	<0.0001

ENS = effective number of species.

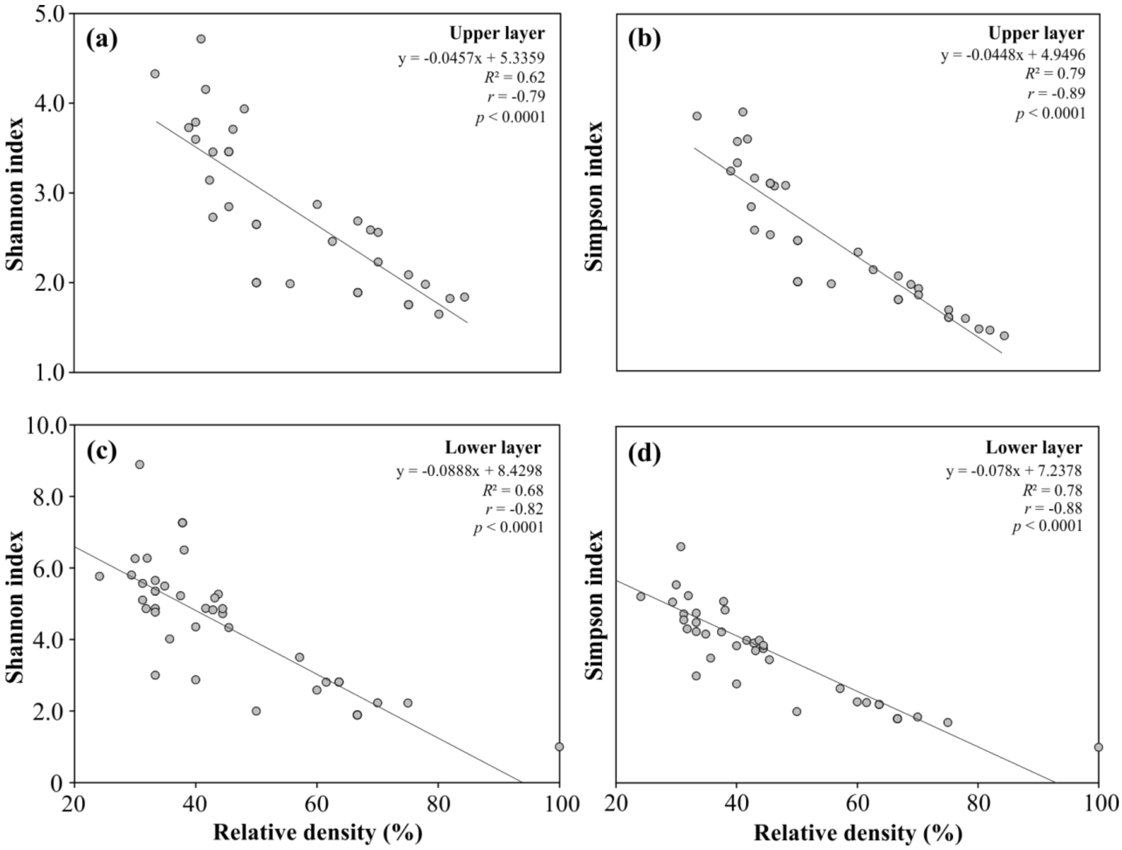

***Figure 4**. Relation between diversity indexes and relative density of the most abundant woody species on upper layer (a, b) and lower (c, d). Each point represents one of 45 sample plots of the natural regeneration of a Subtropical Rainforest in Southern Brazil.*

Discussion

Our study revealed that natural regeneration of Subtropical Rainforest at abandoned pastures is characterized by few highly dominant woody species. These species have specific ecological strategies as stress physiological tolerance and competitive capacity for resources. The ecological strategies of native species are closely related to the successional process (Munoz et al., 2016), where the temporal changes of colonizers and locally persistent and more demanding species are observed at the forest ecosystem affected by a disruption (for example, clearings). In this context, ecological succession

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benefit species with physiological tolerance to stress or with competitive capacity (Caccianiga et al., 2006). Furthermore, the environmental type where the plants are subjected (for example, plains and slopes) can also influence changes at ecological strategies that species have among forest succession. For example, Navas et al. (2010) verified changes between ruderal plants to competitor plants at not stressful ambient and where vital resources are unlimited, whereas at stressful environments and with limited resources ruderal plants tend to be replaced by stress tolerant plants. At the present study, components connected to geomorphology (relief, slope, elevation) and pedology (soil types, water availability) might possibly be acting as environmental filters, as at an environmental gradient relief and soil conditions vary among the slope, enabling influence plants ecological strategies (Thuiller, 2013; Maçaneiro et al., 2016c; Munoz et al., 2016).

Dominant native woody species are common in stressful environments (Peh et al., 2011; Nascimento et al., 2015), like initial successional stage in tropical (Morais et al., 2013) and subtropical rainforests (Klein, 1980; Schorn and Galvão, 2009; Siminski, 2009; Meyer et al., 2013). For example, Steege et al. (2013) determined that Amazon watershed is represented by 16,000 woody species, but that only 227 (1.4%) represent half of all registered woody species. In secondary forests in the initial succession stage, dominant woody species generally are the first plants to grow due to its rapid growth and strong adaptation to local conditions, may formatting dense groupings that characterize young forests canopy (Klein, 1980; 1984; Kageyama and Reis, 1993; Chazdon, 2008; Schorn and Galvão, 2009; Chazdon, 2014; Chazdon and Guariguata, 2016). The establishment of these species during the forest regeneration also contributes to ecosystem resiliency, since they bring mutualist species that generate greater heterogeneity and diversity (Howe, 2016; McAlpine et al., 2016). These species form small patches that provides favorable microclimate for more demanding species grow (Scervino and Torezan, 2015) and attracts seeds dispersing agents, which provides improvement on soil conditions and facilitate forest regeneration (Bechara et al., 2016). Therefore, dominant native woody species generally occur in the initial succession stage of subtropical rainforest and are beneficial for forest regeneration, as observed on this study.

We verified that highly dominant woody species generate lowest natural regeneration diversity, especially on upper layer. In subtropical rainforests, the species located on the upper layer act as environmental filters for those species in lower layer species (see Carvalho et al., 2016; Boukili and Chazdon, 2017). These studies showed that some regenerating woody species are favored by mature species and that the future forest structure is related to the environmental filters. Beyond species, several biotic and abiotic factors act as environmental filters (for example, seeds dispersal, seedlings competition with exotic grasses, predation and germination seedlings, soil chemical and physical characteristics, decaying tree trunks, luminous intensity, herbivory etc), which selected or exclude determined woody species at biological communities (Holl, 2000; Christie and Armesto, 2003; Lortie et al., 2004; Chazdon, 2014; Reid et al., 2015; Chazdon and Guariguata, 2016). However, these environmental filters can help to understand what processes maintain biological diversity and explaining species distribution among environmental gradients (Elith and Leathwick, 2009; Lewis et al., 2014; Maçaneiro et al., 2016c).

Besides that, convert the natural regeneration in a true diversity (effective number of species) will depend on the uniformity vegetation level or on the diversity index

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applied. Considering the characteristics of diversity index in relation to rare species (Magurran, 2004; Jost, 2006; Melo, 2008; Buckland et al., 2011), our study presented that Shannon and Simpson's index are strongly influenced by dominant species. This issue is important when considering the woody species selection for subtropical rainforests regeneration projects, once at the present Brazil suffers with lack environmental legislation and also technical/scientific consistent criteria for species recommendation, leading many projects to use high diversity of species in the forest restoration plantings, what is widely questionable (Naeem, 2006; Wright et al., 2009; Durigan et al., 2010). Our results show that few species can establish themselves in an explored environment by more than half century of intensive use. In this context, implementing actions that provide improvements and facilitate the ecological natural succession processes through dominant native woody species with high density of seedlings are important for the initial stages of subtropical rainforests restauration, since it considers basic principles of "normal" ecological succession (see Suding and Gross, 2006; Naeem, 2006; Durigan and Engel, 2015), besides being an initiative much more affordable, enabling smallholder farmers to restore degraded pastures.

In Neotropical forest restorations, multiple techniques have been used for ecosystem reconstruction with the maximum biodiversity possible, especially planting a high diversity woody species (Rodrigues et al., 2009; Martins, 2013; Bechara et al., 2016; Chazdon and Guariguata, 2016). However, generally the diversity of native species available in tree seedlings nurseries is limited and determined by availability of regional fruits and seeds (Palma and Laurence, 2015; Turchetto et al., 2017). In this study, we observed that some of the most important species are common in all vegetation layers, presenting potential for restoration plantings, especially on open areas as new abandoned pastures, since the are species tolerant to stress and competitors for resources (for example, *Vernonanthura discolor*, *Myrsine coriacea*, *Piptocarpha regnellii*, *Piptocarpha axillaris*, *Piptocarpha angustifolia*, *Clethra scabra* and *Symphyopappus itatiayensis*). Another combination of species that we observed occurs only on low layer, presenting potential for restoration plantings in order to enrichment, at abandoned pasture areas in succession advanced stages (for example, *Ficus luschnathiana*, *Campomanesia guaviroba*, *Inga vera* subsp. *affinis*, *Myrcia splendens*, *Syagrus romanzoffiana*, *Miconia cabucu* and *Alchornea triplinervia*, among several others). Thus, due to structure importance and ecological strategies that these species have in Southern Brazil Subtropical Rainforest natural regeneration, we recommend preferentially this species (or this kind of species) for restoration plantings at abandoned pastures.

In this study, we indicate evidences about how the high dominance of some species (for example, *Vernonanthura discolor*, *Piptocarpha regnellii* and *Myrsine coriacea*) favor the lowest diversity of natural regeneration. However, this standard must be seen as facilitator of forest restoration, once these species presents huge adaptation to local conditions and provide the biggest soil cover and improvement in environmental conditions for more exigent new woody species colonization. These results encourage a further analysis about how these species contribute ecologically for abandoned pastures restorations. Therefore, we suggest the addition of species functional attributes in new vegetation studies, since additional information about ecological strategies of dominant woody species may indicate standards that accelerates the ecological successional process of subtropical rainforests.

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APPENDIX

Appendix 1. *Basic field data 1: species found in upper layer*

Sample plot	Individual	Species	DBH (cm)	Height (m)
PO1	1	Piptocarpha axillaris (Less.) Baker	6,05	2,5
PO1	2	Vernonanthura discolor (Spreng.) H.Rob.	4,93	2,5
PO1	3	Vernonanthura discolor (Spreng.) H.Rob.	4,97	3
PO1	3	Vernonanthura discolor (Spreng.) H.Rob.	5,54	3
PO1	3	Vernonanthura discolor (Spreng.) H.Rob.	5,41	3
PO2	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,41	3
PO2	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,77	3
PO2	2	Vernonanthura discolor (Spreng.) H.Rob.	6,68	4
PO2	2	Vernonanthura discolor (Spreng.) H.Rob.	4,62	4
PO2	3	Vernonanthura discolor (Spreng.) H.Rob.	9,45	5
PO2	3	Vernonanthura discolor (Spreng.) H.Rob.	7,86	5
PO2	4	Vernonanthura discolor (Spreng.) H.Rob.	6,24	2,5
PO3	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,25	2,5
PO3	2	Vernonanthura discolor (Spreng.) H.Rob.	5,25	3
PO4	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,57	3
PO4	2	Piptocarpha axillaris (Less.) Baker	5,09	3
PO4	3	Vernonanthura discolor (Spreng.) H.Rob.	5,41	3
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	4,77	3,5
PO4	5	Piptocarpha angustifolia Dusén ex Malme	5,09	2,5
PO5	1	Piptocarpha angustifolia Dusén ex Malme	8,05	3
PO5	2	Piptocarpha axillaris (Less.) Baker	5,89	3
PO5	3	Piptocarpha axillaris (Less.) Baker	6,53	3,5
PO5	4	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO5	5	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO5	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,93	3
PO5	7	Piptocarpha angustifolia Dusén ex Malme	5,73	3
PO5	8	Piptocarpha angustifolia Dusén ex Malme	5,19	2,5
PO5	9	Piptocarpha angustifolia Dusén ex Malme	5,19	3
PO5	10	Piptocarpha angustifolia Dusén ex Malme	5,09	3
PO5	11	Vernonanthura discolor (Spreng.) H.Rob.	6,05	3,5
PO5	11	Vernonanthura discolor (Spreng.) H.Rob.	5,25	3,5
PO5	11	Vernonanthura discolor (Spreng.) H.Rob.	5,57	3,5
PO6	1	Vernonanthura discolor (Spreng.) H.Rob.	4,77	2,5
PO6	2	Vernonanthura discolor (Spreng.) H.Rob.	5,89	3,5
PO6	3	Piptocarpha axillaris (Less.) Baker	4,77	3
PO6	4	Piptocarpha angustifolia Dusén ex Malme	6,53	3,5
PO6	4	Piptocarpha angustifolia Dusén ex Malme	4,93	3
PO6	5	Piptocarpha axillaris (Less.) Baker	4,77	3
PO6	6	Piptocarpha angustifolia Dusén ex Malme	4,93	3
PO6	7	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO6	8	Vernonanthura discolor (Spreng.) H.Rob.	5,51	3,5
PO6	9	Piptocarpha axillaris (Less.) Baker	4,90	3
PO6	10	Piptocarpha axillaris (Less.) Baker	4,77	3
PO6	11	Piptocarpha angustifolia Dusén ex Malme	7,54	4,5
PO6	12	Vernonanthura discolor (Spreng.) H.Rob.	7,96	4,5

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PO6	13	Vernonanthura discolor (Spreng.) H.Rob.	5,25	3
PO6	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,37	3,5
PO7	1	Vernonanthura discolor (Spreng.) H.Rob.	6,21	3,5
PO7	2	Piptocarpha axillaris (Less.) Baker	6,68	4
PO7	3	Vernonanthura discolor (Spreng.) H.Rob.	6,37	4,5
PO7	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,32	3,5
PO7	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,37	3
PO7	5	Vernonanthura discolor (Spreng.) H.Rob.	6,05	4
PO7	6	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO7	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,92	3,5
PO7	8	Vernonanthura discolor (Spreng.) H.Rob.	4,77	3
PO7	9	Vernonanthura discolor (Spreng.) H.Rob.	6,37	3,5
PO7	10	Vernonanthura discolor (Spreng.) H.Rob.	6,05	4
PO7	10	Vernonanthura discolor (Spreng.) H.Rob.	5,41	4
PO7	11	Vernonanthura discolor (Spreng.) H.Rob.	7,16	4
PO7	12	Vernonanthura discolor (Spreng.) H.Rob.	5,09	4
PO7	13	Vernonanthura discolor (Spreng.) H.Rob.	5,89	3,5
PO7	14	Piptocarpha angustifolia Dusén ex Malme	6,37	4
PO7	15	Vernonanthura discolor (Spreng.) H.Rob.	7,00	4,5
PO7	16	Piptocarpha angustifolia Dusén ex Malme	5,09	3,5
PO8	1	Vernonanthura discolor (Spreng.) H.Rob.	5,41	3
PO8	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,77	3,5
PO8	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,09	3
PO8	3	Piptocarpha angustifolia Dusén ex Malme	5,32	3
PO8	4	Piptocarpha angustifolia Dusén ex Malme	5,63	3
PO8	5	Vernonanthura discolor (Spreng.) H.Rob.	6,21	3
PO8	6	Piptocarpha angustifolia Dusén ex Malme	5,73	4
PO8	6	Piptocarpha angustifolia Dusén ex Malme	5,25	4,5
PO8	7	Vernonanthura discolor (Spreng.) H.Rob.	4,77	3
PO8	8	Piptocarpha angustifolia Dusén ex Malme	9,87	4
PO9	1	Vernonanthura discolor (Spreng.) H.Rob.	5,09	3
PO9	2	Vernonanthura discolor (Spreng.) H.Rob.	5,89	3
PO9	2	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO9	3	Piptocarpha angustifolia Dusén ex Malme	6,37	3,5
PO9	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,53	3
PO9	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,41	3
PO9	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,89	3
PO9	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,21	3
PO9	5	Vernonanthura discolor (Spreng.) H.Rob.	5,09	3,5
PO9	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,96	3,5
PO9	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,68	3,5
PO9	7	Vernonanthura discolor (Spreng.) H.Rob.	5,25	3,5
PO9	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,21	2,5
PO9	9	Piptocarpha axillaris (Less.) Baker	5,73	2,5
PO9	10	Vernonanthura discolor (Spreng.) H.Rob.	5,25	3
PO9	11	Piptocarpha angustifolia Dusén ex Malme	6,37	3
PO10	1	Vernonanthura discolor (Spreng.) H.Rob.	5,09	3,5
PO10	2	Piptocarpha axillaris (Less.) Baker	5,09	3
PO10	3	Piptocarpha angustifolia Dusén ex Malme	8,59	4,5
PO10	3	Piptocarpha angustifolia Dusén ex Malme	7,64	4,5

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PO10	4	Vernonanthura discolor (Spreng.) H.Rob.	6,21	4,5
PO10	4	Vernonanthura discolor (Spreng.) H.Rob.	6,68	4,5
PO10	5	Piptocarpha angustifolia Dusén ex Malme	8,91	4,5
PO10	6	Vernonanthura discolor (Spreng.) H.Rob.	4,93	3
PO10	7	Piptocarpha angustifolia Dusén ex Malme	6,84	3,5
PO10	7	Piptocarpha angustifolia Dusén ex Malme	5,09	3
PO10	8	Piptocarpha angustifolia Dusén ex Malme	4,77	3,5
PO11	1	Vernonanthura discolor (Spreng.) H.Rob.	5,83	3
PO11	2	Piptocarpha axillaris (Less.) Baker	4,77	3
PO11	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,77	2,5
PO11	4	Piptocarpha axillaris (Less.) Baker	4,77	2
PO11	5	Vernonanthura discolor (Spreng.) H.Rob.	6,84	4
PO11	6	Vernonanthura discolor (Spreng.) H.Rob.	8,91	5
PO11	7	Piptocarpha axillaris (Less.) Baker	5,41	4
PO11	7	Piptocarpha axillaris (Less.) Baker	4,77	3,5
PO11	8	Vernonanthura discolor (Spreng.) H.Rob.	4,77	3,5
PO11	9	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	4,93	2,5
PO11	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,73	3
PO12	1	Vernonanthura discolor (Spreng.) H.Rob.	5,25	4
PO13	1	Vernonanthura discolor (Spreng.) H.Rob.	6,75	4,5
PO13	1	Vernonanthura discolor (Spreng.) H.Rob.	5,51	4,5
PO14	1	Vernonanthura discolor (Spreng.) H.Rob.	5,73	3,5
PO15	1	Piptocarpha angustifolia Dusén ex Malme	7,96	5
PO15	1	Piptocarpha angustifolia Dusén ex Malme	5,41	5
PM1	1	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	11,14	5
PM1	1	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	8,28	4
PM1	2	Vernonanthura discolor (Spreng.) H.Rob.	8,91	6
PM1	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,68	6
PM1	4	Vernonanthura discolor (Spreng.) H.Rob.	11,30	6
PM1	4	Vernonanthura discolor (Spreng.) H.Rob.	8,91	6
PM1	5	Vernonanthura discolor (Spreng.) H.Rob.	14,48	8
PM1	5	Vernonanthura discolor (Spreng.) H.Rob.	12,57	8
PM1	6	Vernonanthura discolor (Spreng.) H.Rob.	12,57	4
PM1	7	Vernonanthura discolor (Spreng.) H.Rob.	10,35	6
PM1	7	Vernonanthura discolor (Spreng.) H.Rob.	12,25	7
PM1	7	Vernonanthura discolor (Spreng.) H.Rob.	6,68	6
PM1	7	Vernonanthura discolor (Spreng.) H.Rob.	10,66	6
PM1	8	Vernonanthura discolor (Spreng.) H.Rob.	10,19	6
PM1	9	Vernonanthura discolor (Spreng.) H.Rob.	8,75	6
PM2	1	Annona emarginata (Schltdl.) H.Rainer	8,28	4
PM2	1	Annona emarginata (Schltdl.) H.Rainer	6,37	4
PM2	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,09	5
PM2	3	Vernonanthura discolor (Spreng.) H.Rob.	14,16	8
PM2	4	Annona emarginata (Schltdl.) H.Rainer	9,87	3
PM2	4	Annona emarginata (Schltdl.) H.Rainer	8,28	3
PM2	4	Annona emarginata (Schltdl.) H.Rainer	9,07	3,5
PM2	5	Vernonanthura discolor (Spreng.) H.Rob.	7,00	7
PM2	6	Annona emarginata (Schltdl.) H.Rainer	5,73	3

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PM2	7	Vernonanthura discolor (Spreng.) H.Rob.	14,64	8
PM3	1	Vernonanthura discolor (Spreng.) H.Rob.	6,68	5
PM3	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	8,59	8
PM3	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,21	6
PM3	3	Vernonanthura discolor (Spreng.) H.Rob.	9,07	8
PM3	4	Vernonanthura discolor (Spreng.) H.Rob.	9,23	7
PM3	5	Vernonanthura discolor (Spreng.) H.Rob.	12,10	6
PM3	6	Vernonanthura discolor (Spreng.) H.Rob.	10,66	8
PM3	7	Vernonanthura discolor (Spreng.) H.Rob.	5,73	4
PM3	8	Baccharis semiserrata DC.	6,68	5
PM3	8	Baccharis semiserrata DC.	8,28	4,5
PM3	8	Baccharis semiserrata DC.	5,09	4,5
PM4	1	Vernonanthura discolor (Spreng.) H.Rob.	8,75	8
PM4	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,91	8
PM4	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	7
PM4	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,84	7
PM4	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,93	8
PM4	4	Vernonanthura discolor (Spreng.) H.Rob.	9,23	8
PM4	5	Clethra scabra Pers.	4,93	2,5
PM4	6	Baccharis semiserrata DC.	8,75	4
PM4	6	Baccharis semiserrata DC.	7,80	4
PM4	7	Vernonanthura discolor (Spreng.) H.Rob.	8,59	8
PM4	7	Vernonanthura discolor (Spreng.) H.Rob.	10,98	8
PM4	7	Vernonanthura discolor (Spreng.) H.Rob.	6,37	8
PM4	8	Baccharis semiserrata DC.	7,64	5
PM4	8	Baccharis semiserrata DC.	5,73	5
PM4	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,53	6,5
PM4	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,03	6,5
PM4	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,64	6,5
PM4	10	Baccharis semiserrata DC.	7,00	5
PM4	10	Baccharis semiserrata DC.	6,21	4,5
PM4	10	Baccharis semiserrata DC.	6,37	4
PM4	11	Vernonanthura discolor (Spreng.) H.Rob.	6,05	5
PM4	11	Vernonanthura discolor (Spreng.) H.Rob.	11,65	8
PM4	12	Vernonanthura discolor (Spreng.) H.Rob.	13,05	9
PM5	1	Vernonanthura discolor (Spreng.) H.Rob.	6,05	8
PM5	1	Vernonanthura discolor (Spreng.) H.Rob.	6,84	8
PM5	1	Vernonanthura discolor (Spreng.) H.Rob.	4,77	6
PM5	2	Vernonanthura discolor (Spreng.) H.Rob.	7,32	8
PM5	3	Vernonanthura discolor (Spreng.) H.Rob.	11,62	8
PM5	3	Vernonanthura discolor (Spreng.) H.Rob.	7,96	7
PM5	3	Vernonanthura discolor (Spreng.) H.Rob.	9,39	9
PM5	3	Vernonanthura discolor (Spreng.) H.Rob.	12,73	9
PM5	4	Vernonanthura discolor (Spreng.) H.Rob.	6,37	8
PM5	5	Vernonanthura discolor (Spreng.) H.Rob.	15,60	10
PM5	6	Vernonanthura discolor (Spreng.) H.Rob.	13,05	9
PM5	6	Vernonanthura discolor (Spreng.) H.Rob.	4,77	5
PM5	6	Vernonanthura discolor (Spreng.) H.Rob.	13,21	9
PM5	7	Vernonanthura discolor (Spreng.) H.Rob.	8,91	8
PM5	8	Clethra scabra Pers.	4,93	3,5

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PM5	9	Vernonanthura discolor (Spreng.) H.Rob.	9,87	9
PM5	10	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	6,53	5
PM5	10	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	4,77	5
PM5	11	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	5,09	6
PM5	11	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	4,77	2
PM5	12	Vernonanthura discolor (Spreng.) H.Rob.	4,93	7
PM5	12	Vernonanthura discolor (Spreng.) H.Rob.	7,48	8
PM5	13	Clethra scabra Pers.	5,73	4
PM5	13	Clethra scabra Pers.	5,19	4
PM5	14	Vernonanthura discolor (Spreng.) H.Rob.	15,28	9
PM5	15	Piptocarpha axillaris (Less.) Baker	8,28	7
PM5	15	Piptocarpha axillaris (Less.) Baker	9,23	6
PM6	1	Vernonanthura discolor (Spreng.) H.Rob.	12,89	8
PM6	2	Vernonanthura discolor (Spreng.) H.Rob.	9,55	7
PM6	3	Vernonanthura discolor (Spreng.) H.Rob.	5,09	7
PM6	4	Vernonanthura discolor (Spreng.) H.Rob.	15,44	10
PM6	5	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,82	6
PM6	6	Vernonanthura discolor (Spreng.) H.Rob.	6,84	8
PM6	7	Vernonanthura discolor (Spreng.) H.Rob.	6,68	7
PM6	8	Vernonanthura discolor (Spreng.) H.Rob.	9,07	9
PM6	9	Vernonanthura discolor (Spreng.) H.Rob.	10,82	8
PM6	9	Vernonanthura discolor (Spreng.) H.Rob.	10,03	7
PM6	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,21	5
PM6	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,09	5
PM6	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,89	5
PM6	11	Vernonanthura discolor (Spreng.) H.Rob.	8,91	7
PM6	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,32	6
PM6	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,39	6
PM6	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,37	6
PM6	13	Vernonanthura discolor (Spreng.) H.Rob.	6,53	7
PM6	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,71	6
PM6	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,59	6
PM6	15	Vernonanthura discolor (Spreng.) H.Rob.	10,35	7
PM6	16	Vernonanthura discolor (Spreng.) H.Rob.	6,05	6
PM6	17	Clethra scabra Pers.	5,89	4
PM6	18	Clethra scabra Pers.	4,93	3
PM6	19	Vernonanthura discolor (Spreng.) H.Rob.	6,68	7
PM6	20	Vernonanthura discolor (Spreng.) H.Rob.	7,64	7
PM7	1	Vernonanthura discolor (Spreng.) H.Rob.	12,25	9
PM7	1	Vernonanthura discolor (Spreng.) H.Rob.	10,66	8
PM7	1	Vernonanthura discolor (Spreng.) H.Rob.	9,55	9
PM7	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,50	5
PM7	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	8,12	7
PM7	3	Vernonanthura discolor (Spreng.) H.Rob.	8,12	6
PM7	3	Vernonanthura discolor (Spreng.) H.Rob.	7,96	6
PM7	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	12,41	7
PM7	5	Vernonanthura discolor (Spreng.) H.Rob.	9,87	9

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PM7	6	Vernonanthura discolor (Spreng.) H.Rob.	10,35	9
PM7	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,93	6
PM7	8	Solanum lacerdae Dusén	6,68	4
PM7	9	Vernonanthura discolor (Spreng.) H.Rob.	10,50	7
PM7	10	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,19	4
PM7	11	Vernonanthura discolor (Spreng.) H.Rob.	15,60	9
PM7	11	Vernonanthura discolor (Spreng.) H.Rob.	11,62	8
PM7	12	Clethra scabra Pers.	3,98	4
PM7	12	Clethra scabra Pers.	4,93	4
PM7	13	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	10,98	10
PM8	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,07	5
PM8	2	Vernonanthura discolor (Spreng.) H.Rob.	7,32	9
PM8	2	Vernonanthura discolor (Spreng.) H.Rob.	8,59	9
PM8	2	Vernonanthura discolor (Spreng.) H.Rob.	9,39	9
PM8	3	Vernonanthura discolor (Spreng.) H.Rob.	6,37	9
PM8	4	Vernonanthura discolor (Spreng.) H.Rob.	12,57	10
PM8	4	Vernonanthura discolor (Spreng.) H.Rob.	14,96	10
PM8	5	Vernonanthura discolor (Spreng.) H.Rob.	10,66	8
PM8	5	Vernonanthura discolor (Spreng.) H.Rob.	7,16	8
PM8	6	Vernonanthura discolor (Spreng.) H.Rob.	5,73	7
PM8	7	Vernonanthura discolor (Spreng.) H.Rob.	7,96	9
PM8	8	Vernonanthura discolor (Spreng.) H.Rob.	6,68	9
PM8	9	Vernonanthura discolor (Spreng.) H.Rob.	11,62	10
PM8	9	Vernonanthura discolor (Spreng.) H.Rob.	5,73	7
PM8	10	Vernonanthura discolor (Spreng.) H.Rob.	12,41	10
PM8	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,39	7
PM8	12	Vernonanthura discolor (Spreng.) H.Rob.	18,46	12
PM8	13	Vernonanthura discolor (Spreng.) H.Rob.	9,87	11
PM8	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,48	6
PM8	15	Vernonanthura discolor (Spreng.) H.Rob.	12,57	9
PM8	16	Vernonanthura discolor (Spreng.) H.Rob.	10,82	9
PM8	17	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	7,32	8
PM8	18	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,59	5
PM8	19	Vernonanthura discolor (Spreng.) H.Rob.	6,21	5
PM8	20	Vernonanthura discolor (Spreng.) H.Rob.	5,25	6
PM8	21	Vernonanthura discolor (Spreng.) H.Rob.	3,82	10
PM8	22	Vernonanthura discolor (Spreng.) H.Rob.	6,53	7
PM8	23	Vernonanthura discolor (Spreng.) H.Rob.	5,57	6
PM8	24	Piptocarpha axillaris (Less.) Baker	7,16	6
PM8	25	Piptocarpha axillaris (Less.) Baker	7,00	6
PM8	26	Piptocarpha axillaris (Less.) Baker	12,41	8
PM8	27	Piptocarpha axillaris (Less.) Baker	9,55	8
PM8	28	Vernonanthura discolor (Spreng.) H.Rob.	5,09	6
PM8	29	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,07	7
PM8	30	Vernonanthura discolor (Spreng.) H.Rob.	9,23	9
PM8	31	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,50	8
PM8	32	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,91	8
PM8	33	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,68	9
PM8	34	Vernonanthura discolor (Spreng.) H.Rob.	11,46	12
PM8	34	Vernonanthura discolor (Spreng.) H.Rob.	6,05	8

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PM8	35	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,09	6
PM9	1	Vernonanthura discolor (Spreng.) H.Rob.	8,12	8
PM9	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,44	7
PM9	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,89	5
PM9	4	Vernonanthura discolor (Spreng.) H.Rob.	7,64	9
PM9	5	Vernonanthura discolor (Spreng.) H.Rob.	9,07	9
PM9	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	11,62	7
PM9	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,16	5
PM9	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	4,5
PM9	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,44	6
PM9	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,84	7
PM9	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,05	6
PM9	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,41	7
PM9	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,57	6
PM9	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,41	8
PM9	12	Vernonanthura discolor (Spreng.) H.Rob.	6,68	8
PM9	13	Vernonanthura discolor (Spreng.) H.Rob.	7,16	8
PM9	13	Vernonanthura discolor (Spreng.) H.Rob.	6,05	7
PM9	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,48	8
PM9	15	Vernonanthura discolor (Spreng.) H.Rob.	5,09	6
PM9	15	Vernonanthura discolor (Spreng.) H.Rob.	7,96	6
PM9	15	Vernonanthura discolor (Spreng.) H.Rob.	5,89	5
PM9	16	Vernonanthura discolor (Spreng.) H.Rob.	6,68	8
PM9	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,48	6
PM9	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,64	6
PM9	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,80	6
PM9	18	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,59	7
PM9	19	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	6
PM9	20	Vernonanthura discolor (Spreng.) H.Rob.	4,93	6
PM9	21	Vernonanthura discolor (Spreng.) H.Rob.	10,19	9
PM9	22	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,57	5
PM9	23	Vernonanthura discolor (Spreng.) H.Rob.	7,32	7
PM9	24	Vernonanthura discolor (Spreng.) H.Rob.	5,57	6
PM9	25	Vernonanthura discolor (Spreng.) H.Rob.	6,53	7
PM9	26	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,07	8
PM9	27	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,19	7
PM9	28	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,77	7
PM9	29	Piptocarpha axillaris (Less.) Baker	8,59	7
PM9	29	Piptocarpha axillaris (Less.) Baker	7,64	7
PM9	30	Vernonanthura discolor (Spreng.) H.Rob.	7,48	8
PM9	30	Vernonanthura discolor (Spreng.) H.Rob.	9,87	8
PM9	30	Vernonanthura discolor (Spreng.) H.Rob.	9,07	8
PM9	31	Vernonanthura discolor (Spreng.) H.Rob.	9,39	8
PM9	32	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,80	7
PM9	33	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,53	7
PM9	33	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,68	7
PM9	33	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,48	7
PM10	1	Vernonanthura discolor (Spreng.) H.Rob.	7,00	8
PM10	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,98	7
PM10	3	Vernonanthura discolor (Spreng.) H.Rob.	4,77	4

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PM10	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,50	6
PM10	5	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,89	5
PM10	6	Baccharis semiserrata DC.	5,41	5
PM10	7	Baccharis semiserrata DC.	7,96	6
PM10	7	Baccharis semiserrata DC.	8,12	6
PM10	8	Vernonanthura discolor (Spreng.) H.Rob.	7,96	5
PM10	9	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	4,97	5
PM10	9	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	5,09	5
PM10	10	Vernonanthura discolor (Spreng.) H.Rob.	5,47	4
PM10	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,25	4
PM10	12	Piptocarpha axillaris (Less.) Baker	4,93	5
PM10	12	Piptocarpha axillaris (Less.) Baker	4,93	5
PM10	13	Piptocarpha axillaris (Less.) Baker	8,12	6
PM10	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	4,5
PM10	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,80	6
PM10	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,68	5
PM10	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,44	7
PM10	15	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,09	3,5
PM10	16	Vernonanthura discolor (Spreng.) H.Rob.	6,84	7
PM10	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,64	6
PM10	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,89	7
PM10	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	9,17	7
PM10	18	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,84	6
PM10	19	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	6
PM10	19	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,37	6
PM10	20	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,03	6
PM10	21	Piptocarpha axillaris (Less.) Baker	5,09	5
PM10	22	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,41	4
PM11	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,32	7
PM11	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	18,46	8
PM11	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	12,57	8
PM11	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	12,73	8
PM11	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,77	4
PM11	5	Vernonanthura discolor (Spreng.) H.Rob.	6,05	8
PM11	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,91	8
PM11	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,21	6
PM11	8	Piptocarpha angustifolia Dusén ex Malme	8,98	8
PM11	8	Piptocarpha angustifolia Dusén ex Malme	7,32	8
PM11	9	Vernonanthura discolor (Spreng.) H.Rob.	5,73	8
PM11	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,50	7
PM11	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,32	6
PM11	12	Vernonanthura discolor (Spreng.) H.Rob.	5,89	9
PM11	13	Ocotea odorifera (Vell.) Rohwer	17,19	6
PM11	13	Ocotea odorifera (Vell.) Rohwer	12,83	6
PM11	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,41	9
PM11	15	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,73	5
PM11	16	Vernonanthura discolor (Spreng.) H.Rob.	4,93	5
PM11	17	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,75	7

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PM11	18	Piptocarpha regnellii (Sch.Bip.) Cabrera	12,10	9
PM11	19	Vernonanthura discolor (Spreng.) H.Rob.	8,91	8
PM11	20	Piptocarpha angustifolia Dusén ex Malme	9,87	8
PM11	20	Piptocarpha angustifolia Dusén ex Malme	10,19	8
PM11	21	Vernonanthura discolor (Spreng.) H.Rob.	5,41	8
PM11	22	Piptocarpha axillaris (Less.) Baker	6,21	8
PM11	22	Piptocarpha axillaris (Less.) Baker	4,93	8
PM11	23	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,77	7
PM11	24	Vernonanthura discolor (Spreng.) H.Rob.	14,01	10
PM11	25	Piptocarpha regnellii (Sch.Bip.) Cabrera	14,64	8
PM11	25	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	8
PM11	25	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,57	6
PM12	1	Vernonanthura discolor (Spreng.) H.Rob.	9,55	8
PM12	2	Clethra scabra Pers.	4,93	5
PM12	3	Vernonanthura discolor (Spreng.) H.Rob.	7,64	5
PM12	4	Vernonanthura discolor (Spreng.) H.Rob.	10,50	7
PM12	5	Vernonanthura discolor (Spreng.) H.Rob.	7,70	7
PM12	6	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	11,78	5
PM12	7	Vernonanthura discolor (Spreng.) H.Rob.	8,28	8
PM12	7	Vernonanthura discolor (Spreng.) H.Rob.	6,84	8
PM12	8	Vernonanthura discolor (Spreng.) H.Rob.	7,19	7
PM12	9	Vernonanthura discolor (Spreng.) H.Rob.	11,62	8
PM12	10	Baccharis semiserrata DC.	7,00	5
PM13	1	Baccharis semiserrata DC.	8,28	5
PM13	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	11,78	6
PM13	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,80	4
PM13	3	Vernonanthura discolor (Spreng.) H.Rob.	5,41	5
PM13	4	Vernonanthura discolor (Spreng.) H.Rob.	8,12	7
PM13	5	Vernonanthura discolor (Spreng.) H.Rob.	8,75	6
PM13	6	Vernonanthura discolor (Spreng.) H.Rob.	9,87	7,5
PM13	7	Vernonanthura discolor (Spreng.) H.Rob.	7,70	6
PM13	7	Vernonanthura discolor (Spreng.) H.Rob.	7,32	6
PM13	7	Vernonanthura discolor (Spreng.) H.Rob.	6,91	6
PM13	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,86	4
PM13	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,28	6
PM13	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,96	5
PM14	-	-	-	-
PM15	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	5,73	3,5
PM15	2	Vernonanthura discolor (Spreng.) H.Rob.	8,09	7
PM15	3	Vernonanthura discolor (Spreng.) H.Rob.	5,73	7
PM15	3	Vernonanthura discolor (Spreng.) H.Rob.	8,12	7
PM15	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,59	7
PM15	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,60	7
PM15	5	Vernonanthura discolor (Spreng.) H.Rob.	8,12	7
PM15	5	Vernonanthura discolor (Spreng.) H.Rob.	9,01	7
PM15	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,68	5
PM15	7	Vernonanthura discolor (Spreng.) H.Rob.	10,98	8
PM15	8	Vernonanthura discolor (Spreng.) H.Rob.	10,66	6,5
PM15	8	Vernonanthura discolor (Spreng.) H.Rob.	8,59	6,5

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PM15	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	11,87	7,5
R1	1	Vernonanthura discolor (Spreng.) H.Rob.	7,48	6
R1	2	Vernonanthura discolor (Spreng.) H.Rob.	9,23	7
R1	3	Vernonanthura discolor (Spreng.) H.Rob.	9,71	7
R1	3	Vernonanthura discolor (Spreng.) H.Rob.	8,12	6
R1	4	Vernonanthura discolor (Spreng.) H.Rob.	5,09	4
R1	5	Vernonanthura discolor (Spreng.) H.Rob.	8,91	6
R1	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	11,78	5
R1	7	Vernonanthura discolor (Spreng.) H.Rob.	9,07	7
R1	7	Vernonanthura discolor (Spreng.) H.Rob.	6,21	5
R1	7	Vernonanthura discolor (Spreng.) H.Rob.	7,32	6
R1	8	Vernonanthura discolor (Spreng.) H.Rob.	6,05	4
R1	8	Vernonanthura discolor (Spreng.) H.Rob.	10,66	7
R1	9	Vernonanthura discolor (Spreng.) H.Rob.	4,84	4
R1	9	Vernonanthura discolor (Spreng.) H.Rob.	4,93	4
R1	9	Vernonanthura discolor (Spreng.) H.Rob.	4,93	4
R1	10	Piptocarpha axillaris (Less.) Baker	5,57	3
R1	11	Vernonanthura discolor (Spreng.) H.Rob.	10,19	6
R2	1	Vernonanthura discolor (Spreng.) H.Rob.	8,75	7
R2	1	Vernonanthura discolor (Spreng.) H.Rob.	5,25	5
R2	1	Vernonanthura discolor (Spreng.) H.Rob.	5,57	6
R2	2	Piptocarpha axillaris (Less.) Baker	5,16	4
R2	3	Vernonanthura discolor (Spreng.) H.Rob.	9,80	8
R2	3	Vernonanthura discolor (Spreng.) H.Rob.	11,78	8
R2	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,11	4
R2	5	Vernonanthura discolor (Spreng.) H.Rob.	7,96	5
R2	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	7,32	7
R2	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,05	6
R2	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,16	5
R2	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	4,93	4
R2	9	Aspidosperma tomentosum Mart.	6,05	3,5
R3	1	Vernonanthura discolor (Spreng.) H.Rob.	5,41	5
R3	2	Vernonanthura discolor (Spreng.) H.Rob.	5,25	5
R3	3	Vernonanthura discolor (Spreng.) H.Rob.	6,84	7
R3	4	Vernonanthura discolor (Spreng.) H.Rob.	6,05	7
R3	5	Vernonanthura discolor (Spreng.) H.Rob.	7,64	6
R4	1	Piptocarpha axillaris (Less.) Baker	8,69	7
R4	1	Piptocarpha axillaris (Less.) Baker	7,00	7
R4	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,75	6
R4	3	Piptocarpha axillaris (Less.) Baker	8,12	7
R4	4	Vernonanthura discolor (Spreng.) H.Rob.	10,82	10
R4	5	Piptocarpha axillaris (Less.) Baker	5,67	6
R4	6	Piptocarpha axillaris (Less.) Baker	8,91	7
R4	6	Piptocarpha axillaris (Less.) Baker	6,37	6
R4	7	Vernonanthura discolor (Spreng.) H.Rob.	7,16	8
R4	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,05	7
R4	9	Piptocarpha axillaris (Less.) Baker	5,09	4
R4	10	Vernonanthura discolor (Spreng.) H.Rob.	7,86	8
R4	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,46	8
R4	12	Vernonanthura discolor (Spreng.) H.Rob.	7,73	7

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R4	13	Piptocarpha axillaris (Less.) Baker	5,79	4,5
R4	14	Vernonanthura discolor (Spreng.) H.Rob.	5,25	6
R4	15	Vernonanthura discolor (Spreng.) H.Rob.	5,89	8
R4	16	Vernonanthura discolor (Spreng.) H.Rob.	8,44	10
R4	16	Vernonanthura discolor (Spreng.) H.Rob.	8,28	10
R4	17	Piptocarpha axillaris (Less.) Baker	8,75	7
R4	18	Ocotea puberula (Rich.) Nees	5,57	5
R4	19	Vernonanthura discolor (Spreng.) H.Rob.	8,59	10
R4	20	Piptocarpha axillaris (Less.) Baker	9,17	10
R4	21	Vernonanthura discolor (Spreng.) H.Rob.	15,12	10
R4	22	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	10,44	9
R4	23	Piptocarpha axillaris (Less.) Baker	6,68	5
R4	24	Piptocarpha axillaris (Less.) Baker	7,48	7
R4	25	Vernonanthura discolor (Spreng.) H.Rob.	9,07	9
R4	26	Piptocarpha axillaris (Less.) Baker	6,05	5
R5	1	Vernonanthura discolor (Spreng.) H.Rob.	6,84	7
R5	2	Vernonanthura discolor (Spreng.) H.Rob.	15,76	10
R5	2	Vernonanthura discolor (Spreng.) H.Rob.	12,67	10
R5	3	Vernonanthura discolor (Spreng.) H.Rob.	5,16	4
R5	3	Vernonanthura discolor (Spreng.) H.Rob.	8,28	7
R5	3	Vernonanthura discolor (Spreng.) H.Rob.	8,91	8
R5	4	Vernonanthura discolor (Spreng.) H.Rob.	6,68	7
R5	5	Clethra scabra Pers.	7,16	5
R5	6	Vernonanthura discolor (Spreng.) H.Rob.	12,10	9
R5	6	Vernonanthura discolor (Spreng.) H.Rob.	7,16	8
R5	7	Vernonanthura discolor (Spreng.) H.Rob.	10,66	7
R5	8	Vernonanthura discolor (Spreng.) H.Rob.	10,82	9
R5	9	Vernonanthura discolor (Spreng.) H.Rob.	9,87	7
R5	10	Vernonanthura discolor (Spreng.) H.Rob.	9,87	9
R5	11	Vernonanthura discolor (Spreng.) H.Rob.	6,53	8
R5	12	Vernonanthura discolor (Spreng.) H.Rob.	9,80	9
R5	13	Piptocarpha axillaris (Less.) Baker	9,93	8
R5	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,05	8
R5	15	Vernonanthura discolor (Spreng.) H.Rob.	11,62	9
R5	16	Vernonanthura discolor (Spreng.) H.Rob.	9,93	10
R5	16	Vernonanthura discolor (Spreng.) H.Rob.	5,41	8
R5	17	Vernonanthura discolor (Spreng.) H.Rob.	8,47	10
R5	18	Vernonanthura discolor (Spreng.) H.Rob.	8,21	10
R5	18	Vernonanthura discolor (Spreng.) H.Rob.	10,82	10
R5	19	Vernonanthura discolor (Spreng.) H.Rob.	7,32	6
R5	19	Vernonanthura discolor (Spreng.) H.Rob.	13,05	9
R6	1	Piptocarpha axillaris (Less.) Baker	4,84	4
R6	2	Piptocarpha axillaris (Less.) Baker	8,05	7
R6	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,40	6
R6	4	Piptocarpha axillaris (Less.) Baker	6,68	7
R6	5	Vernonanthura discolor (Spreng.) H.Rob.	9,87	9
R6	5	Vernonanthura discolor (Spreng.) H.Rob.	8,12	8
R6	6	Piptocarpha axillaris (Less.) Baker	5,25	5
R6	7	Vernonanthura discolor (Spreng.) H.Rob.	6,43	7
R6	8	Vernonanthura discolor (Spreng.) H.Rob.	5,73	7

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R6	9	Piptocarpha axillaris (Less.) Baker	5,41	5
R6	10	Piptocarpha axillaris (Less.) Baker	5,89	7
R6	11	Vernonanthura discolor (Spreng.) H.Rob.	5,89	6
R6	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	8,28	6
R6	13	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,84	6
R6	14	Vernonanthura discolor (Spreng.) H.Rob.	6,65	6
R6	15	Vernonanthura discolor (Spreng.) H.Rob.	6,11	5
R6	16	Piptocarpha axillaris (Less.) Baker	9,87	8
R6	17	Piptocarpha axillaris (Less.) Baker	5,35	5
R6	17	Piptocarpha axillaris (Less.) Baker	6,68	5
R6	18	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,84	6
R6	18	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	8,75	10
R6	19	Piptocarpha angustifolia Dusén ex Malme	6,43	6
R6	19	Piptocarpha angustifolia Dusén ex Malme	8,91	6
R6	20	Vernonanthura discolor (Spreng.) H.Rob.	7,64	7
R6	20	Vernonanthura discolor (Spreng.) H.Rob.	6,37	6
R6	21	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	5
R6	21	Piptocarpha regnellii (Sch.Bip.) Cabrera	7,00	6
R6	21	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,35	6
R6	22	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5,73	8
R6	23	Vernonanthura discolor (Spreng.) H.Rob.	8,28	7
R6	24	Piptocarpha axillaris (Less.) Baker	7,70	6
R6	25	Vernonanthura discolor (Spreng.) H.Rob.	6,81	5
R6	26	Piptocarpha axillaris (Less.) Baker	4,77	4
R6	27	Piptocarpha axillaris (Less.) Baker	6,68	6
R6	28	Vernonanthura discolor (Spreng.) H.Rob.	5,73	5
R6	29	Piptocarpha axillaris (Less.) Baker	6,02	5
R6	30	Piptocarpha regnellii (Sch.Bip.) Cabrera	10,89	7
R6	31	Vernonanthura discolor (Spreng.) H.Rob.	9,23	7
R6	31	Vernonanthura discolor (Spreng.) H.Rob.	4,93	4
R6	31	Vernonanthura discolor (Spreng.) H.Rob.	7,96	6
R6	32	Piptocarpha axillaris (Less.) Baker	7,64	5
R6	33	Vernonanthura discolor (Spreng.) H.Rob.	6,37	6
R6	34	Vernonanthura discolor (Spreng.) H.Rob.	6,53	6
R6	35	Piptocarpha regnellii (Sch.Bip.) Cabrera	6,37	5
R6	36	Piptocarpha axillaris (Less.) Baker	5,57	5
R6	36	Piptocarpha axillaris (Less.) Baker	5,57	4
R7	1	Piptocarpha axillaris (Less.) Baker	7,00	4
R7	2	Vernonanthura discolor (Spreng.) H.Rob.	7,00	4
R7	2	Vernonanthura discolor (Spreng.) H.Rob.	5,09	4
R7	3	Vernonanthura discolor (Spreng.) H.Rob.	8,75	6
R8	1	Vernonanthura discolor (Spreng.) H.Rob.	6,46	5
R8	2	Ocotea puberula (Rich.) Nees	5,57	3
R8	3	Vernonanthura discolor (Spreng.) H.Rob.	7,32	5
R8	4	Vernonanthura discolor (Spreng.) H.Rob.	6,84	6
R8	5	Vernonanthura discolor (Spreng.) H.Rob.	7,16	6
R8	5	Vernonanthura discolor (Spreng.) H.Rob.	9,39	7
R8	5	Vernonanthura discolor (Spreng.) H.Rob.	9,17	5
R9	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,77	3,5
R10	1	Vernonanthura discolor (Spreng.) H.Rob.	5,41	4

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R10	2	Vernonanthura discolor (Spreng.) H.Rob.	10,03	6
R10	2	Vernonanthura discolor (Spreng.) H.Rob.	8,15	6
R11	-	-	-	-
R12	1	Vernonanthura discolor (Spreng.) H.Rob.	5,09	4,5
R12	2	Ocotea puberula (Rich.) Nees	7,00	3
R13	1	Vernonanthura discolor (Spreng.) H.Rob.	5,09	5
R13	2	Vernonanthura discolor (Spreng.) H.Rob.	7,80	7
R13	3	Vernonanthura discolor (Spreng.) H.Rob.	6,53	6
R13	3	Vernonanthura discolor (Spreng.) H.Rob.	7,64	6
R13	4	Clethra scabra Pers.	4,87	3
R14	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6,37	5
R15	1	Vernonanthura discolor (Spreng.) H.Rob.	4,93	4
R15	1	Vernonanthura discolor (Spreng.) H.Rob.	4,93	4
R15	2	Baccharis dracunculifolia DC.	6,53	5
R15	2	Baccharis dracunculifolia DC.	4,93	5
R15	2	Baccharis dracunculifolia DC.	5,16	5

Appendix 2. *Basic field data 2: species found in lower layer*

Sample plot	Individual	Species	Height (m)
PO1	1	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO1	2	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO1	3	Piptocarpha angustifolia Dusén ex Malme	3
PO1	3	Piptocarpha angustifolia Dusén ex Malme	3
PO1	4	Piptocarpha axillaris (Less.) Baker	2,5
PO1	4	Piptocarpha axillaris (Less.) Baker	2,5
PO1	5	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO1	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,7
PO1	7	Vernonanthura discolor (Spreng.) H.Rob.	1,1
PO1	8	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,8
PO1	9	Piptocarpha axillaris (Less.) Baker	1,3
PO1	10	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO1	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,9
PO1	12	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2,5
PO1	13	Vernonanthura discolor (Spreng.) H.Rob.	2
PO1	14	Ficus luschnathiana (Miq.) Miq.	1,1
PO2	1	Vernonanthura discolor (Spreng.) H.Rob.	3
PO2	2	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO2	2	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO2	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO2	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO2	3	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO2	4	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PO2	5	Piptocarpha axillaris (Less.) Baker	0,8
PO2	6	Baccharis dracunculifolia DC.	1,5
PO2	7	Leandra glazioviana Cogn.	0,6
PO2	8	Vernonanthura discolor (Spreng.) H.Rob.	1,8

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PO2	9	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO2	10	Vernonanthura discolor (Spreng.) H.Rob.	0,7
PO2	11	Piptocarpha angustifolia Dusén ex Malme	1,5
PO2	12	Piptocarpha angustifolia Dusén ex Malme	2
PO2	13	Baccharis oblongifolia (Ruiz & Pav.) Pers.	2
PO2	14	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO2	14	Vernonanthura discolor (Spreng.) H.Rob.	2
PO2	14	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO2	15	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PO2	16	Piptocarpha angustifolia Dusén ex Malme	3
PO2	17	Piptocarpha axillaris (Less.) Baker	2,5
PO2	18	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,5
PO2	19	Piptocarpha axillaris (Less.) Baker	2
PO2	20	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PO2	21	Solanum pseudoquina A.St.-Hil.	2
PO2	21	Solanum pseudoquina A.St.-Hil.	2
PO2	22	Clethra scabra Pers.	0,7
PO2	23	Piptocarpha angustifolia Dusén ex Malme	3
PO2	24	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO2	25	Vernonanthura discolor (Spreng.) H.Rob.	3
PO2	26	Vernonanthura discolor (Spreng.) H.Rob.	2
PO2	27	Piptocarpha axillaris (Less.) Baker	0,8
PO2	28	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO2	29	Clethra scabra Pers.	1,3
PO2	30	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO2	31	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO2	32	Vernonanthura discolor (Spreng.) H.Rob.	3
PO2	33	Solanum pseudoquina A.St.-Hil.	1,9
PO2	33	Solanum pseudoquina A.St.-Hil.	1,9
PO2	34	Vernonanthura discolor (Spreng.) H.Rob.	3
PO2	35	Jacaranda puberula Cham.	1
PO2	36	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PO2	37	Baccharis oblongifolia (Ruiz & Pav.) Pers.	1,8
PO3	1	Vernonanthura discolor (Spreng.) H.Rob.	2
PO3	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO3	3	Vernonanthura discolor (Spreng.) H.Rob.	2,1
PO3	4	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO3	5	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO3	6	Vernonanthura discolor (Spreng.) H.Rob.	3
PO3	7	Vernonanthura discolor (Spreng.) H.Rob.	3
PO3	8	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,8
PO3	9	Vernonanthura discolor (Spreng.) H.Rob.	1,3
PO3	10	Vernonanthura discolor (Spreng.) H.Rob.	1
PO3	11	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO3	12	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO3	13	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO3	14	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO3	15	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO3	15	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5

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PO3	16	Vernonanthura discolor (Spreng.) H.Rob.	2
PO3	17	Vernonanthura discolor (Spreng.) H.Rob.	2
PO3	18	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO3	19	Miconia sellowiana Naudin	1,5
PO3	20	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO4	1	Myrsine umbellata Mart.	1,3
PO4	2	Vernonanthura discolor (Spreng.) H.Rob.	1,9
PO4	3	Piptocarpha axillaris (Less.) Baker	2
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	1
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	1
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PO4	4	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PO4	5	Myrsine umbellata Mart.	1
PO4	6	Myrsine umbellata Mart.	0,6
PO4	7	Myrsine umbellata Mart.	0,9
PO4	8	Myrsine umbellata Mart.	0,8
PO4	9	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	10	Vernonanthura discolor (Spreng.) H.Rob.	1
PO4	11	Piptocarpha angustifolia Dusén ex Malme	2,5
PO4	12	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1
PO4	13	Vernonanthura discolor (Spreng.) H.Rob.	1,6
PO4	14	Baccharis dracunculifolia DC.	2,5
PO4	15	Piptocarpha axillaris (Less.) Baker	1,6
PO4	16	Piptocarpha axillaris (Less.) Baker	2,5
PO4	16	Piptocarpha axillaris (Less.) Baker	2,5
PO4	17	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	17	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	18	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	18	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	18	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	18	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO4	19	Solanum lacerdae Dusén	2,5
PO4	20	Piptocarpha angustifolia Dusén ex Malme	1,5
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	0,5
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	1
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	0,7
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	21	Vernonanthura discolor (Spreng.) H.Rob.	2
PO4	22	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO4	23	Piptocarpha regnellii (Sch.Bip.) Cabrera	1
PO4	24	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,8
PO4	24	Piptocarpha regnellii (Sch.Bip.) Cabrera	2

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PO4	25	Piptocarpha angustifolia Dusén ex Malme	1
PO4	26	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO4	26	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	1	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	2	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO5	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	3	Clethra scabra Pers.	0,8
PO5	4	Piptocarpha axillaris (Less.) Baker	3
PO5	5	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	6	Clethra scabra Pers.	2
PO5	7	Handroanthus chrysotrichus (Mart. ex DC.) Mattos	0,6
PO5	8	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO5	9	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO5	9	Vernonanthura discolor (Spreng.) H.Rob.	2
PO5	10	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2
PO5	11	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2
PO5	12	Vernonanthura discolor (Spreng.) H.Rob.	3
PO5	13	Piptocarpha angustifolia Dusén ex Malme	2,5
PO5	14	Piptocarpha axillaris (Less.) Baker	2
PO5	15	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO5	16	Ficus luschnathiana (Miq.) Miq.	2
PO6	1	Piptocarpha angustifolia Dusén ex Malme	2,5
PO6	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PO6	3	Clethra scabra Pers.	1
PO6	4	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO6	5	Zanthoxylum rhoifolium Lam.	1,3
PO6	6	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO6	7	Vernonanthura discolor (Spreng.) H.Rob.	2
PO6	8	Piptocarpha angustifolia Dusén ex Malme	0,8
PO6	9	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO6	10	Zanthoxylum rhoifolium Lam.	1,8
PO6	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO6	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO6	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,8
PO6	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO6	13	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO6	14	Piptocarpha angustifolia Dusén ex Malme	3,5
PO6	15	Clethra scabra Pers.	0,6
PO6	16	Vernonanthura discolor (Spreng.) H.Rob.	0,7
PO6	17	Vernonanthura discolor (Spreng.) H.Rob.	1,3
PO6	18	Vernonanthura discolor (Spreng.) H.Rob.	3
PO6	19	Piptocarpha angustifolia Dusén ex Malme	3
PO6	19	Piptocarpha angustifolia Dusén ex Malme	3
PO6	19	Piptocarpha angustifolia Dusén ex Malme	2
PO6	20	Piptocarpha angustifolia Dusén ex Malme	2,5
PO6	21	Piptocarpha axillaris (Less.) Baker	2
PO6	22	Piptocarpha angustifolia Dusén ex Malme	1
PO6	23	Vernonanthura discolor (Spreng.) H.Rob.	1,5

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PO6	24	Solanum mauritianum Scop.	0,7
PO7	1	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO7	2	Vernonanthura discolor (Spreng.) H.Rob.	4
PO7	2	Vernonanthura discolor (Spreng.) H.Rob.	4
PO7	2	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	2	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO7	3	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	3	Vernonanthura discolor (Spreng.) H.Rob.	3
PO7	4	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	4	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	4	Vernonanthura discolor (Spreng.) H.Rob.	3
PO7	5	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO7	5	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	6	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO7	7	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PO7	8	Clethra scabra Pers.	2
PO7	9	Piptocarpha axillaris (Less.) Baker	3
PO7	9	Piptocarpha axillaris (Less.) Baker	3,5
PO7	10	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,8
PO7	11	Myrsine umbellata Mart.	1,8
PO7	12	Myrsine umbellata Mart.	0,8
PO7	13	Myrsine umbellata Mart.	1
PO7	13	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO7	14	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO7	15	Piptocarpha angustifolia Dusén ex Malme	3
PO7	16	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	3
PO7	17	Piptocarpha axillaris (Less.) Baker	1,5
PO7	18	Clethra scabra Pers.	0,8
PO8	1	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO8	1	Vernonanthura discolor (Spreng.) H.Rob.	3
PO8	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PO8	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PO8	3	Vernonanthura discolor (Spreng.) H.Rob.	2
PO8	4	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2,5
PO8	5	Solanum mauritianum Scop.	2,5
PO8	6	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2
PO8	7	Vernonanthura discolor (Spreng.) H.Rob.	3
PO8	8	Baccharis dracunculifolia DC.	1,5
PO8	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO8	10	Vernonanthura discolor (Spreng.) H.Rob.	2
PO8	11	Piptocarpha angustifolia Dusén ex Malme	2,5
PO8	12	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO8	12	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO8	13	Piptocarpha angustifolia Dusén ex Malme	2,5
PO8	14	Miconia inconspicua Miq.	1,8
PO8	15	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO8	16	Piptocarpha axillaris (Less.) Baker	0,7

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PO8	17	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	3,5
PO8	18	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO8	19	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PO8	19	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PO8	20	Piptocarpha axillaris (Less.) Baker	2,5
PO8	21	Piptocarpha angustifolia Dusén ex Malme	1,5
PO9	1	Solanum variabile Mart.	2
PO9	2	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO9	3	Piptocarpha axillaris (Less.) Baker	2
PO9	3	Piptocarpha axillaris (Less.) Baker	1,8
PO9	4	Baccharis semiserrata DC.	3
PO9	4	Baccharis semiserrata DC.	3
PO9	5	Vernonanthura discolor (Spreng.) H.Rob.	2
PO9	6	Vernonanthura discolor (Spreng.) H.Rob.	3
PO9	6	Vernonanthura discolor (Spreng.) H.Rob.	3
PO9	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,5
PO9	8	Piptocarpha axillaris (Less.) Baker	2,5
PO9	9	Piptocarpha axillaris (Less.) Baker	1
PO9	10	Solanum lacerdae Dusén	2
PO9	11	Piptocarpha axillaris (Less.) Baker	2
PO9	12	Vernonanthura discolor (Spreng.) H.Rob.	2
PO9	13	Vernonanthura discolor (Spreng.) H.Rob.	2
PO9	14	Vernonanthura discolor (Spreng.) H.Rob.	2,3
PO9	15	Piptocarpha axillaris (Less.) Baker	2
PO9	16	Vernonanthura discolor (Spreng.) H.Rob.	0,7
PO9	17	Vernonanthura discolor (Spreng.) H.Rob.	2
PO9	17	Vernonanthura discolor (Spreng.) H.Rob.	2
PO9	18	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO9	19	Vernonanthura discolor (Spreng.) H.Rob.	1
PO9	20	Vernonanthura discolor (Spreng.) H.Rob.	3
PO9	20	Vernonanthura discolor (Spreng.) H.Rob.	3
PO9	20	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO9	21	Baccharis semiserrata DC.	2,5
PO9	21	Baccharis semiserrata DC.	2,5
PO9	22	Baccharis semiserrata DC.	3
PO10	1	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,5
PO10	2	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO10	3	Piptocarpha angustifolia Dusén ex Malme	2,5
PO10	4	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO10	5	Vernonanthura discolor (Spreng.) H.Rob.	1,6
PO10	6	Solanum mauritianum Scop.	2
PO10	6	Solanum mauritianum Scop.	2
PO10	7	Vernonanthura discolor (Spreng.) H.Rob.	2
PO10	8	Vernonanthura discolor (Spreng.) H.Rob.	3
PO10	9	Piptocarpha angustifolia Dusén ex Malme	2
PO10	10	Vernonanthura discolor (Spreng.) H.Rob.	2
PO10	11	Baccharis semiserrata DC.	3
PO10	12	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	3

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PO10	12	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	2,5
PO10	13	Vernonanthura discolor (Spreng.) H.Rob.	3
PO10	13	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO10	13	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO10	14	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PO11	2	Vernonanthura discolor (Spreng.) H.Rob.	1,8
PO11	3	Annona emarginata (Schltdl.) H.Rainer	0,6
PO11	4	Piptocarpha axillaris (Less.) Baker	2,5
PO11	5	Piptocarpha axillaris (Less.) Baker	1,5
PO11	6	Vernonanthura discolor (Spreng.) H.Rob.	2
PO11	7	Piptocarpha axillaris (Less.) Baker	2,5
PO11	8	Piptocarpha axillaris (Less.) Baker	2,5
PO11	9	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	9	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	9	Vernonanthura discolor (Spreng.) H.Rob.	2
PO11	9	Vernonanthura discolor (Spreng.) H.Rob.	2
PO11	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO11	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,5
PO11	10	Piptocarpha regnellii (Sch.Bip.) Cabrera	1
PO11	11	Piptocarpha axillaris (Less.) Baker	2
PO11	12	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	13	Baccharis semiserrata DC.	3,5
PO11	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PO11	14	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,5
PO11	15	Piptocarpha axillaris (Less.) Baker	3
PO11	16	Piptocarpha regnellii (Sch.Bip.) Cabrera	1
PO11	17	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	17	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	17	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	17	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	18	Piptocarpha axillaris (Less.) Baker	1,8
PO11	19	Vernonanthura discolor (Spreng.) H.Rob.	3
PO11	19	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	20	Piptocarpha axillaris (Less.) Baker	3
PO11	21	Piptocarpha axillaris (Less.) Baker	2,5
PO11	22	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	3
PO11	23	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	24	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	24	Vernonanthura discolor (Spreng.) H.Rob.	2
PO11	24	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO11	24	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO11	25	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO11	26	Piptocarpha axillaris (Less.) Baker	1
PO11	27	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO11	28	Baccharis semiserrata DC.	5
PO11	28	Baccharis semiserrata DC.	2,5
PO11	29	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	30	Vernonanthura discolor (Spreng.) H.Rob.	1,8

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PO11	31	Piptocarpha angustifolia Dusén ex Malme	2
PO11	32	Piptocarpha axillaris (Less.) Baker	2
PO11	32	Piptocarpha axillaris (Less.) Baker	2
PO11	32	Piptocarpha axillaris (Less.) Baker	1,5
PO11	33	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO11	34	Baccharis oblongifolia (Ruiz & Pav.) Pers.	2,5
PO11	34	Baccharis oblongifolia (Ruiz & Pav.) Pers.	2
PO11	34	Baccharis oblongifolia (Ruiz & Pav.) Pers.	2
PO11	35	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,8
PO11	36	Clethra scabra Pers.	1,8
PO11	36	Clethra scabra Pers.	1,8
PO11	37	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,5
PO11	38	Vernonanthura discolor (Spreng.) H.Rob.	1
PO11	39	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO11	40	Piptocarpha axillaris (Less.) Baker	3,5
PO11	40	Piptocarpha axillaris (Less.) Baker	3,5
PO11	41	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PO11	42	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PO11	43	Vernonanthura discolor (Spreng.) H.Rob.	1
PO12	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8
PO12	2	Baccharis semiserrata DC.	1,8
PO12	3	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PO12	4	Vernonanthura discolor (Spreng.) H.Rob.	2
PO12	5	Vernonanthura discolor (Spreng.) H.Rob.	1
PO12	6	Piptocarpha axillaris (Less.) Baker	1,8
PO12	6	Piptocarpha axillaris (Less.) Baker	1,5
PO12	7	Piptocarpha axillaris (Less.) Baker	2
PO12	7	Piptocarpha axillaris (Less.) Baker	1,8
PO12	8	Clethra scabra Pers.	0,5
PO12	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	1
PO12	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	1
PO12	10	Vernonanthura discolor (Spreng.) H.Rob.	0,5
PO12	11	Baccharis semiserrata DC.	1,5
PO12	12	Clethra scabra Pers.	0,6
PO12	13	Myrsine umbellata Mart.	0,5
PO12	14	Baccharis semiserrata DC.	4
PO12	14	Baccharis semiserrata DC.	4
PO12	14	Baccharis semiserrata DC.	4
PO12	14	Baccharis semiserrata DC.	4
PO12	14	Baccharis semiserrata DC.	4
PO12	14	Baccharis semiserrata DC.	4
PO12	15	Baccharis semiserrata DC.	4
PO12	16	Clethra scabra Pers.	1
PO12	17	Baccharis semiserrata DC.	4
PO12	17	Baccharis semiserrata DC.	4
PO12	17	Baccharis semiserrata DC.	4
PO12	17	Baccharis semiserrata DC.	4
PO12	17	Baccharis semiserrata DC.	3
PO12	17	Baccharis semiserrata DC.	3
PO12	17	Baccharis semiserrata DC.	3

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PO12	17	Baccharis semiserrata DC.	3
PO13	1	Myrsine umbellata Mart.	1
PO13	2	Baccharis semiserrata DC.	3
PO13	3	Piptocarpha axillaris (Less.) Baker	3
PO13	3	Piptocarpha axillaris (Less.) Baker	2,5
PO13	4	Piptocarpha angustifolia Dusén ex Malme	1,7
PO13	5	Vernonanthura discolor (Spreng.) H.Rob.	1,7
PO13	6	Myrsine umbellata Mart.	1
PO14	1	Solanum mauritianum Scop.	1,8
PO14	2	Piptocarpha angustifolia Dusén ex Malme	1,8
PO14	3	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PO15	1	Baccharis semiserrata DC.	4
PO15	2	Piptocarpha angustifolia Dusén ex Malme	3,5
PM1	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM1	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM1	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM1	3	Syagrus romanzoffiana (Cham.) Glassman	1,8
PM1	4	Miconia sellowiana Naudin	1,8
PM1	5	Myrcia splendens (Sw.) DC.	0,6
PM1	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
PM1	7	Myrcia splendens (Sw.) DC.	0,8
PM1	8	Myrcia splendens (Sw.) DC.	0,5
PM1	9	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
PM1	10	Inga vera subsp. affinis (DC.) T.D.Penn.	0,5
PM1	11	Vernonanthura discolor (Spreng.) H.Rob.	0,5
PM1	12	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PM1	13	Vernonanthura discolor (Spreng.) H.Rob.	5
PM1	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PM1	15	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PM1	16	Vernonanthura discolor (Spreng.) H.Rob.	4
PM2	1	Vernonanthura discolor (Spreng.) H.Rob.	1
PM2	2	Campomanesia reitziana D.Legrand	0,5
PM2	3	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM2	4	Clethra scabra Pers.	3
PM2	4	Clethra scabra Pers.	3
PM2	5	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM2	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PM2	7	Syagrus romanzoffiana (Cham.) Glassman	0,8
PM2	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM2	9	Syagrus romanzoffiana (Cham.) Glassman	0,5
PM2	10	Inga vera subsp. affinis (DC.) T.D.Penn.	0,8
PM2	11	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM2	12	Clethra scabra Pers.	3
PM3	1	Miconia tristis Spring	0,8
PM3	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM3	3	Miconia sellowiana Naudin	0,6
PM3	4	Miconia tristis Spring	0,6
PM3	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PM3	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM3	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3

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PM3	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM3	9	Solanum lacerdae Dusén	3
PM3	10	Clethra scabra Pers.	2,5
PM3	10	Clethra scabra Pers.	2,5
PM3	11	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM3	12	Vernonanthura discolor (Spreng.) H.Rob.	0,5
PM4	1	Clethra scabra Pers.	3
PM4	1	Clethra scabra Pers.	3
PM4	1	Clethra scabra Pers.	3
PM4	2	Vernonanthura discolor (Spreng.) H.Rob.	2
PM4	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM4	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM4	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM4	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PM4	6	Vernonanthura discolor (Spreng.) H.Rob.	2
PM4	7	Vernonanthura discolor (Spreng.) H.Rob.	2
PM4	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM4	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM4	10	Clethra scabra Pers.	3
PM4	11	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PM4	12	Dalbergia brasiliensis Vogel	2
PM4	13	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM4	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM5	1	Clethra scabra Pers.	2,5
PM5	1	Clethra scabra Pers.	2,5
PM5	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM5	3	Vernonanthura discolor (Spreng.) H.Rob.	2
PM5	4	Clethra scabra Pers.	3
PM5	4	Clethra scabra Pers.	3
PM5	4	Clethra scabra Pers.	3
PM5	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM5	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM5	7	Leandra carassana (DC.) Cogn.	1,5
PM5	8	Miconia tristis Spring	0,6
PM5	9	Miconia tristis Spring	0,8
PM5	10	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
PM5	11	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PM5	12	Miconia tristis Spring	0,6
PM5	13	Miconia tristis Spring	0,8
PM5	14	Miconia tristis Spring	1
PM5	15	Clethra scabra Pers.	2
PM5	16	Piptocarpha regnellii (Sch.Bip.) Cabrera	3,5
PM5	17	Nectandra oppositifolia Nees	0,6
PM5	18	Vernonanthura discolor (Spreng.) H.Rob.	1
PM5	19	Miconia tristis Spring	0,6
PM5	20	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM5	21	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM5	21	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM5	22	Clethra scabra Pers.	1,5
PM5	23	Vernonanthura discolor (Spreng.) H.Rob.	1

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PM5	24	Vernonanthura discolor (Spreng.) H.Rob.	4
PM5	25	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM5	26	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM5	27	Miconia tristis Spring	4
PM5	28	Vernonanthura discolor (Spreng.) H.Rob.	2
PM5	29	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM6	1	Myrsine umbellata Mart.	4
PM6	1	Myrsine umbellata Mart.	4
PM6	1	Myrsine umbellata Mart.	4
PM6	2	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PM6	3	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PM6	4	Inga vera subsp. affinis (DC.) T.D.Penn.	0,8
PM6	5	Clethra scabra Pers.	3
PM6	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	5
PM6	7	Miconia tristis Spring	0,7
PM6	8	Clethra scabra Pers.	3,5
PM6	9	Vernonanthura discolor (Spreng.) H.Rob.	5
PM7	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM7	2	Vernonanthura discolor (Spreng.) H.Rob.	1
PM7	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1
PM7	4	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM7	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM7	6	Miconia sellowiana Naudin	1,5
PM7	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
PM7	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,3
PM7	9	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM7	10	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8
PM7	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8
PM7	12	Vernonanthura discolor (Spreng.) H.Rob.	0,6
PM7	13	Vernonanthura discolor (Spreng.) H.Rob.	3
PM8	1	Ficus luschnathiana (Miq.) Miq.	3
PM8	2	Guatteria australis A.St.-Hil.	1,8
PM8	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM8	4	Alchornea triplinervia (Spreng.) Müll.Arg.	0,8
PM8	5	Piptocarpha axillaris (Less.) Baker	2
PM8	6	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM8	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM8	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM8	9	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM8	10	Annona emarginata (Schltdl.) H.Rainer	0,6
PM8	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5
PM8	12	Campomanesia guaviroba (DC.) Kiaersk.	1,5
PM8	13	Clethra scabra Pers.	4
PM8	14	Clethra scabra Pers.	3
PM8	14	Clethra scabra Pers.	3
PM8	14	Clethra scabra Pers.	3
PM8	15	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM8	16	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM8	17	Myrcia splendens (Sw.) DC.	2,5
PM8	18	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4

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PM8	19	Piptocarpha regnellii (Sch.Bip.) Cabrera	4
PM8	20	Vernonanthura discolor (Spreng.) H.Rob.	5,5
PM8	21	Piptocarpha regnellii (Sch.Bip.) Cabrera	4
PM8	22	Piptocarpha axillaris (Less.) Baker	2,5
PM8	23	Piptocarpha axillaris (Less.) Baker	2
PM8	24	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM8	25	Clethra scabra Pers.	3,5
PM8	26	Miconia cabucu Hoehne	1,5
PM9	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,8
PM9	2	Miconia tristis Spring	0,6
PM9	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM9	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4,5
PM9	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1
PM9	6	Vernonanthura discolor (Spreng.) H.Rob.	2,5
PM9	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM9	8	Miconia tristis Spring	0,6
PM9	9	Miconia cabucu Hoehne	1,8
PM9	10	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PM10	1	Vernonanthura discolor (Spreng.) H.Rob.	1,5
PM10	2	Clethra scabra Pers.	3
PM10	2	Clethra scabra Pers.	3
PM10	3	Clethra scabra Pers.	1
PM10	4	Clethra scabra Pers.	0,8
PM10	5	Clethra scabra Pers.	3
PM10	5	Clethra scabra Pers.	3
PM10	5	Clethra scabra Pers.	3
PM10	5	Clethra scabra Pers.	3
PM10	6	Ocotea puberula (Rich.) Nees	1,6
PM10	7	Piptocarpha axillaris (Less.) Baker	1,5
PM10	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM10	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	3
PM10	10	Clethra scabra Pers.	3
PM10	10	Clethra scabra Pers.	3
PM10	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	6
PM10	12	Miconia sellowiana Naudin	1
PM10	13	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5
PM10	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
PM10	15	Piptocarpha regnellii (Sch.Bip.) Cabrera	5
PM11	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	2,5
PM11	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,9
PM11	3	Vernonanthura discolor (Spreng.) H.Rob.	3
PM11	4	Miconia sellowiana Naudin	0,7
PM11	5	Piptocarpha regnellii (Sch.Bip.) Cabrera	3,5
PM11	6	Clethra scabra Pers.	3,5
PM11	6	Clethra scabra Pers.	3,5
PM11	6	Clethra scabra Pers.	3,5
PM11	7	Clethra scabra Pers.	0,7
PM11	8	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM11	9	Vernonanthura discolor (Spreng.) H.Rob.	0,8
PM11	10	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3

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PM11	11	Piptocarpha regnellii (Sch.Bip.) Cabrera	3,5
PM11	12	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM11	13	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
PM11	14	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PM11	15	Vernonanthura discolor (Spreng.) H.Rob.	2
PM11	16	Myrsine umbellata Mart.	1
PM12	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	0,8
PM12	2	Clethra scabra Pers.	1
PM12	3	Rubus brasiliensis Mart.	2
PM12	4	Vernonanthura discolor (Spreng.) H.Rob.	3
PM12	5	Piptocarpha axillaris (Less.) Baker	1
PM12	6	Baccharis semiserrata DC.	4
PM12	6	Baccharis semiserrata DC.	4
PM12	6	Baccharis semiserrata DC.	4
PM12	6	Baccharis semiserrata DC.	4
PM12	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	1,2
PM12	8	Baccharis semiserrata DC.	2,5
PM12	8	Baccharis semiserrata DC.	2,5
PM12	9	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PM12	10	Vernonanthura discolor (Spreng.) H.Rob.	3
PM12	10	Vernonanthura discolor (Spreng.) H.Rob.	3
PM12	11	Clethra scabra Pers.	2
PM12	12	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PM12	13	Alchornea triplinervia (Spreng.) Müll.Arg.	1
PM12	14	Baccharis oblongifolia (Ruiz & Pav.) Pers.	0,7
PM13	1	Piptocarpha regnellii (Sch.Bip.) Cabrera	2
PM13	2	Vernonanthura discolor (Spreng.) H.Rob.	1
PM13	3	Clethra scabra Pers.	3,5
PM13	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM13	5	Vernonanthura discolor (Spreng.) H.Rob.	3
PM13	6	Annona emarginata (Schltdl.) H.Rainer	1,5
PM13	7	Miconia tristis Spring	1
PM13	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
PM13	9	Vernonanthura discolor (Spreng.) H.Rob.	4,5
PM13	10	Baccharis semiserrata DC.	4
PM14	1	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,7
PM14	1	Symphyopappus itatiayensis (Hieron.) R.M.King & H.Rob.	1,4
PM15	1	Baccharis semiserrata DC.	3,5
PM15	1	Baccharis semiserrata DC.	3,5
PM15	1	Baccharis semiserrata DC.	3,5
PM15	1	Baccharis semiserrata DC.	3
PM15	1	Baccharis semiserrata DC.	3
PM15	1	Baccharis semiserrata DC.	3
PM15	2	Miconia tristis Spring	0,5
PM15	3	Miconia tristis Spring	0,5
PM15	4	Miconia tristis Spring	0,5
PM15	5	Vernonanthura discolor (Spreng.) H.Rob.	3,5
PM15	5	Vernonanthura discolor (Spreng.) H.Rob.	2
PM15	6	Vernonanthura discolor (Spreng.) H.Rob.	1,8

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PM15	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	4
PM15	7	Piptocarpha regnellii (Sch.Bip.) Cabrera	4
PM15	8	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM15	9	Vernonanthura discolor (Spreng.) H.Rob.	4
PM15	10	Clethra scabra Pers.	3,5
PM15	11	Vernonanthura discolor (Spreng.) H.Rob.	2
PM15	12	Vernonanthura discolor (Spreng.) H.Rob.	5
PM15	13	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
PM15	14	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
PM15	15	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2
R1	1	Vernonanthura discolor (Spreng.) H.Rob.	3
R1	2	Vernonanthura discolor (Spreng.) H.Rob.	1
R1	3	Myrsine umbellata Mart.	2,5
R1	4	Miconia lymanii Wurdack	2
R1	4	Miconia lymanii Wurdack	2
R1	4	Miconia lymanii Wurdack	2
R1	5	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R1	6	Vernonanthura discolor (Spreng.) H.Rob.	1,5
R1	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8
R1	8	Clethra scabra Pers.	1,5
R1	9	Critoniopsis quinqueflora (Less.) H.Rob.	1,8
R2	1	Vernonanthura discolor (Spreng.) H.Rob.	2
R2	2	Vernonanthura discolor (Spreng.) H.Rob.	4
R2	3	Clethra scabra Pers.	2,5
R2	3	Clethra scabra Pers.	2,5
R2	3	Clethra scabra Pers.	2,5
R2	4	Clethra scabra Pers.	2,5
R2	4	Clethra scabra Pers.	2,5
R2	5	Cedrela fissilis Vell.	1,5
R3	1	Ocotea puberula (Rich.) Nees	3
R3	2	Vernonanthura discolor (Spreng.) H.Rob.	3
R3	3	Vernonanthura discolor (Spreng.) H.Rob.	3
R3	4	Vernonanthura discolor (Spreng.) H.Rob.	3
R3	5	Clethra scabra Pers.	2,5
R3	5	Clethra scabra Pers.	2,5
R3	5	Clethra scabra Pers.	2,5
R4	1	Vernonanthura discolor (Spreng.) H.Rob.	1,8
R4	2	Vernonanthura discolor (Spreng.) H.Rob.	2
R4	3	Vernonanthura discolor (Spreng.) H.Rob.	5
R4	4	Vernonanthura discolor (Spreng.) H.Rob.	5
R4	5	Vernonanthura discolor (Spreng.) H.Rob.	3
R4	5	Vernonanthura discolor (Spreng.) H.Rob.	3,5
R4	6	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R4	7	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R4	8	Piptocarpha axillaris (Less.) Baker	3,5
R4	9	Piptocarpha regnellii (Sch.Bip.) Cabrera	4
R4	10	Piptocarpha axillaris (Less.) Baker	5
R5	1	Cyathea phalerata Mart.	2
R5	2	Vernonanthura discolor (Spreng.) H.Rob.	4
R5	3	Myrsine umbellata Mart.	1,6

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R5	4	Vernonanthura discolor (Spreng.) H.Rob.	0,7
R5	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,7
R5	6	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R5	7	Vernonanthura discolor (Spreng.) H.Rob.	4
R5	8	Vernonanthura discolor (Spreng.) H.Rob.	4
R5	9	Vernonanthura discolor (Spreng.) H.Rob.	1
R5	10	Cyathea phalerata Mart.	1
R5	11	Vernonanthura discolor (Spreng.) H.Rob.	5
R6	1	Vernonanthura discolor (Spreng.) H.Rob.	4
R6	1	Vernonanthura discolor (Spreng.) H.Rob.	3
R6	1	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R6	2	Piptocarpha axillaris (Less.) Baker	3
R6	2	Piptocarpha axillaris (Less.) Baker	3
R6	3	Myrsine umbellata Mart.	4,5
R6	4	Ocotea elegans Mez	1
R6	5	Vernonanthura discolor (Spreng.) H.Rob.	4
R6	6	Piptocarpha axillaris (Less.) Baker	4,5
R6	7	Piptocarpha angustifolia Dusén ex Malme	4
R6	8	Piptocarpha axillaris (Less.) Baker	4
R6	9	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
R6	10	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,8
R6	11	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
R6	12	Ocotea elegans Mez	0,6
R6	13	Piptocarpha axillaris (Less.) Baker	4
R6	14	Vernonanthura discolor (Spreng.) H.Rob.	6
R6	15	Vernonanthura discolor (Spreng.) H.Rob.	4
R6	16	Vernonanthura discolor (Spreng.) H.Rob.	3
R6	17	Piptocarpha axillaris (Less.) Baker	3
R6	18	Ocotea elegans Mez	1,5
R6	19	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5
R6	20	Piptocarpha axillaris (Less.) Baker	3
R6	21	Piptocarpha axillaris (Less.) Baker	3
R6	22	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R7	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
R7	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
R7	3	Ocotea puberula (Rich.) Nees	1
R7	4	Ocotea puberula (Rich.) Nees	1
R8	-	-	-
R9		Solanum mauritianum Scop.	3,5
R10	1	Miconia tristis Spring	0,6
R10	2	Clethra scabra Pers.	1
R10	2	Clethra scabra Pers.	1
R10	3	Vernonanthura discolor (Spreng.) H.Rob.	1
R10	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,6
R10	5	Miconia tristis Spring	0,5
R10	6	Clethra scabra Pers.	1,5
R10	7	Clethra scabra Pers.	1
R10	8	Vernonanthura discolor (Spreng.) H.Rob.	1,5
R10	9	Miconia tristis Spring	0,5
R10	10	Clethra scabra Pers.	1,8

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R10	12	Clethra scabra Pers.	1,2
R10	13	Clethra scabra Pers.	0,5
R10	14	Clethra scabra Pers.	1,2
R10	15	Ocotea puberula (Rich.) Nees	1,2
R10	16	Solanum pseudoquina A.St.-Hil.	1
R10	17	Solanum pseudoquina A.St.-Hil.	0,5
R10	18	Solanum pseudoquina A.St.-Hil.	1
R10	19	Solanum pseudoquina A.St.-Hil.	1,2
R10	20	Casearia sylvestris Sw.	1,2
R10	21	Clethra scabra Pers.	2
R10	22	Solanum mauritianum Scop.	1,2
R10	23	Solanum pseudoquina A.St.-Hil.	1
R10	24	Solanum pseudoquina A.St.-Hil.	1
R10	25	Ocotea puberula (Rich.) Nees	0,7
R10	26	Casearia sylvestris Sw.	1,7
R10	27	Clethra scabra Pers.	1,8
R10	27	Clethra scabra Pers.	1,8
R10	27	Clethra scabra Pers.	1,8
R10	28	Miconia tristis Spring	0,8
R10	29	Solanum pseudoquina A.St.-Hil.	0,5
R10	30	Solanum pseudoquina A.St.-Hil.	1
R10	31	Solanum pseudoquina A.St.-Hil.	0,5
R10	32	Solanum pseudoquina A.St.-Hil.	0,6
R10	33	Solanum pseudoquina A.St.-Hil.	0,7
R10	34	Solanum pseudoquina A.St.-Hil.	0,8
R10	35	Solanum pseudoquina A.St.-Hil.	0,8
R10	36	Solanum pseudoquina A.St.-Hil.	0,7
R10	37	Solanum pseudoquina A.St.-Hil.	0,5
R10	38	Solanum pseudoquina A.St.-Hil.	0,5
R10	39	Solanum pseudoquina A.St.-Hil.	0,7
R10	40	Solanum pseudoquina A.St.-Hil.	0,7
R10	41	Solanum pseudoquina A.St.-Hil.	0,8
R10	42	Solanum pseudoquina A.St.-Hil.	0,8
R10	43	Solanum pseudoquina A.St.-Hil.	0,8
R10	44	Solanum pseudoquina A.St.-Hil.	0,8
R10	45	Campomanesia guaviroba (DC.) Kiaersk.	2
R10	46	Campomanesia guaviroba (DC.) Kiaersk.	1,8
R10	47	Campomanesia guaviroba (DC.) Kiaersk.	1,8
R10	48	Clethra scabra Pers.	1,8
R10	49	Clethra scabra Pers.	1,8
R10	50	Clethra scabra Pers.	1,6
R10	51	Clethra scabra Pers.	1,5
R10	52	Miconia tristis Spring	0,6
R11	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8
R11	2	Aspidosperma tomentosum Mart.	1,5
R11	3	Aspidosperma tomentosum Mart.	1,8
R12	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	4
R12	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
R13	1	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,5
R13	2	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	1,8

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R13	3	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	0,9
R13	4	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	5
R13	5	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3,5
R13	6	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	2,5
R13	7	Myrsine coriacea (Sw.) R.Br. ex Roem. & Schult.	3
R13	8	Miconia tristis Spring	0,8
R13	9	Clethra scabra Pers.	0,6
R13	10	Clethra scabra Pers.	0,6
R13	11	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R14	1	Vernonanthura discolor (Spreng.) H.Rob.	2,5
R15	1	Vernonanthura discolor (Spreng.) H.Rob.	4
R15	2	Miconia tristis Spring	1
R15	3	Miconia tristis Spring	1

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EFFECTS OF MANAGEMENT PRACTICES IN HIGHLAND PASTURES ON AGRONOMIC AND ENVIRONMENTAL OBJECTIVES

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Abstract. Resolution of plant diversity goals with the agricultural demands placed on grasslands may result in conflict between farmers and conservationists. To investigate options for reconciling these important issues implementable in the short-term within agro-environmental schemes, we analysed the impact of four years' management with grazing replaced by mowing on coordination of agricultural objectives, i.e. herbage yield, plant functional groups, nutrient contents and removal, forage value and plant diversity, i.e. species richness, Shannon-Wiener index, Ellenberg N indicator value. The study was carried out in three hillslope highland pasture sites (S1-S3) in the Šumava Mountains, Czech Republic, differing in soil properties and animal stocking rates, which were 0.92 in S1, 0.34 in S2, combined with mowing in some years and 0.73 in S3 LU ha^{-1}. Some sections of each pasture site were twice yearly mowed. Our findings related to grazing combined or replaced with mowing were relevant to farmers' priorities acceptable for dairy cattle nutrition. Across sites, the herbage dry matter yield, herbage P, K and Mg contents and removal, proportion of graminoids and Ellenberg N values decreased with mowing, but forage value and herbage Ca removal remained identical under both managements. Species richness, Shannon-Wiener index and herbage Ca content increased with mowing via spread of forbs and/or legumes. However, to achieve a merit, short-term grazing exclusion with mowing has to take into account site-specific soil nutrient conditions. Very high soil P availability prevented from the increase in species richness with mowing (S3). Contrarily, in sites with lower soil P availability, but different availability of other nutrients (S1, S2), mowing fostered number and/or proportions of forbs and/or legumes.
Keywords: *grazing, mowing, grassland, available soil nutrients, biodiversity, above-ground biomass*

Introduction

Resolution of plant diversity goals with the agricultural demands placed on grasslands is an important issue of agri-environmental policies (Isselstein et al., 2005). Improvement in plant diversity (species richness and species evenness) is linked with extensive grassland management, which is often accompanied by a decline in agricultural production (Plantureux et al., 2005; Blüthgen et al., 2012). Hence, plant species diversity, forage quality, and biomass production may not correlate, resulting in conflict between farmers and conservationists (Pavlů et al., 2006). To resolve these issues, an effective agro-environment subsidy policy is needed to compensate for production losses. In addition, positive effects of vegetation diversity on production

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stability, efficient nutrient and water use, and forage quality may encourage farmers to cooperate (Sanderson et al., 2004; Wrage et al., 2011).

The plant diversity-productivity relationship varies strongly along with site conditions and is integrated in herbage nutrient contents, which reflect plant growth, plant functional composition, land use, and management practices (Klaus et al., 2013a).

Extensive grazing, the predominant farming system in upland and marginal areas, can deliver both economic benefits and increase in grassland biodiversity, reducing the environmental impact of animal production (Marriott et al., 2005; Rose et al., 2012). Plant relations in pastures influence biodiversity responses through both positive (e.g., facilitation, N_2 fixation, hydraulic lift) and negative interactions (e.g., competitive exclusion, allelopathy) (Sanderson et al., 2004). Grazing at a stocking rate (SR) of fewer than 1.5 livestock units (LU) ha^{-1} has the potential to facilitate restoration of sward heterogeneity and thus floral and faunal diversity, by selective defoliation through grazing, treading, nutrient cycling, and propagule dispersal (Zhang et al., 2013; Dubeux et al., 2014; Quan et al., 2015). A variety of vegetation structures can develop regardless soil conditions under the influence of grazing animals and their foraging strategy resulting to structural patterns of short- and high-growing vegetation patches (Gilhaus et al., 2014). Short-growing species with higher fodder values are preferably grazed and, hence, the likelihood of their endozoochorous dispersion is supposed (Gilhaus et al., 2017).

To restore plant diversity in soils with intermediate nutrient content, mowing of grazed pastures has been demonstrated to reduce nutrient availability, especially phosphorus (Isselstein et al., 2005; Blüthgen et al., 2012). Alternating mowing and grazing should promote coexistence of multiple forbs and grasses and balance nutrient limitation (Mládková et al., 2015). Plantureux et al. (2005) considered the combination of an early cut and late grazing as a compromise between quality forage production and biodiversity maintenance for the conditions of Central Europe. A combination of grazing and mowing can also enhance the opportunity for regeneration of seedlings, if a closed canopy exists in grazed plant communities (Plantureux et al., 2005).

Our study was conducted in three highland pasture sites to investigate options for reconciling plant diversity and agronomy targets implementable in the short-term within agro-environmental schemes. We evaluated the effects of four years' mowing as opposed to grazing on agronomic factors and plant diversity parameters. Prior to our research, we hypothesized that i) short-term mowing could improve plant diversity parameters (species richness, Shannon-Wiener index, and Ellenberg N indicator), but ii) eventually deteriorate agronomic objectives (herbage dry matter yield, plant functional groups, herbage nutrient content and removal, tetany ratio, and forage value). However, a complex of site nutrient conditions allow specific adaptations to management changes (Gilhaus and Hölzel, 2016). Hence, we also took into account the applicability of short-term grazing exclusion with mowing to plant diversity improvement related to different soil site conditions and grazing types.

Materials and methods

Study site

The study took place 2011-2014 in Pasečná (48°36'18.0"N; 14°07'12.0"E), an upland, geomorphologically heterogeneous, plateau near the Czech-Austrian border, in the foothills of the Šumava Mountains (*Fig. 1*). Mean altitude of the study site is 822.6 m a.s.l. with range from 781.4 to 882.4 m a.s.l. The region is cool with mean air

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temperatures annually and in the growing season of 5.5 °C and 11.2 °C, respectively, and average annual and growing season precipitation of 910 mm and 510 mm, respectively. Parent rock in Pasečná is granite. The soils are predominantly acidic, Haplic to Gleyed Cambisols, and Gleysols with distinct stand-conditioned dissimilarities in soil texture, soil depth, and soil water regime. In 1993, the study area changed from intensive cultivation to pasture. Until 2011, the land was under a rotational pasturing with mean SR of 0.7 LU ha^{-1}, with the lowest SR in S2 due to the combination with occasional mowing.

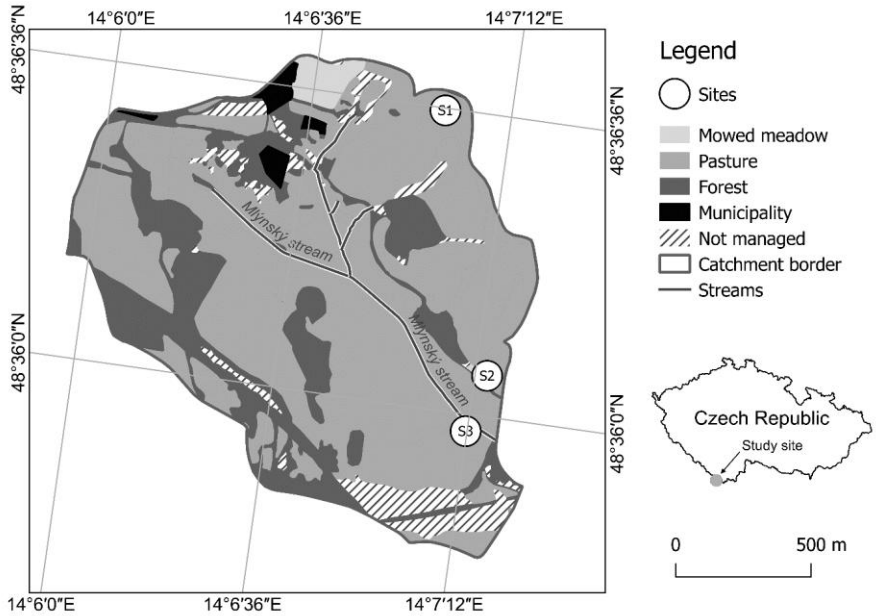

Figure 1. *The catchment in Pasečná; experimental sites S1-S3*

Three experimental sites differing in soil properties and SR (*Table 1*) were selected randomly in hillslope geomorphological zones (recharge – S1, transport – S2, discharge – S3). Site S3 consisted of tile-drained soil.

Table 1. *Site characteristics, management, stocking rate, plant cover and plant species (2011-2014)*

Site	S1		S2		S3	
Altitude	837 m a.s.l		797 m a.s.l		786 m a.s.l	
Soil type	Leptic Cambisol, loam-sand		Haplic Cambisol, sand-loam		Haplic Gleysol, loam-sand	
Management	Mowed	Grazed (SR)	Mowed	Grazed combined with mowed (SR)	Mowed	Grazed (SR)
2011	TM	TRG (0.76)	TM	SM + AG (0.40)	TM	TRG (0.50)
2012	TM	TRG (0.88)	TM	CG (0.56)	TM	CG (0.56)
2013	TM	TRG (0.95)	TM	TM	TM	CG (0.93)
2014	TM	TRG (1.1)	TM	SM + TRG (0.41)	TM	CG (0.93)

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Mean SR		0.92		0.34		0.73
Mean cover of vascular plants (%), G:L:F	86 55:1:30	84 64:4:16	84 50:11:23	79 56:8:15	89 73:1:15	85 66:7:12
Total N G:L:F	41 11:1:29	34 11:3:20	45 12:6:27	44 11:6:27	39 15:3:21	35 13:2:20
Dominant G (%)	*Dactylis glomerata* (23) *Lolium perenne* (14.9) *Poa pratensis* (10.5)	*D. glomerata* (31.8) *L. perenne* (15.4) *P. pratensis* (6.3) *Poa trivialis* (5.6)	*Festuca. rubra* (14.7) *Agrostis capillaris* (14.6) *P. pratensis* (8.8) *D. glomerata* (6.3)	*A. capillaris* (25.1) *F. rubra* (9.9) *P. pratensis* (7.6) *D. glomerata* (7.1)	*A. capillaris* (33.9) *P. pratensis* (21.8) *D. glomerata* (7.5)	*A. capillaris* (41.7) *P. pratensis* (15.2)
Dominant L (%)	*Trifolium repens* (1.1)	*T. repens* (3.5)	*T. repens* (4.7) *Trifolium pratense* (4.2)	*T. repens* (4) *T. pratense* (2.4)	*Lathyrus pratensis* (0.6)	*T. repens* (6.5)
Dominant F (%)	*Anthriscus sylvestris* (8.6) *Heracleum sphondylium* (6.9), *Taraxacum sect. ruderalia* (2.6), *Pimpinella major* (2.5), *Achillea millefolium* (2.4), *Veronica chamaedrys* (2.3)	*T. sect. ruderalia* (9.4)	*T. sect. ruderalia* (4) *A. sylvestris* (2.7) *Hypericum maculatum* (2.6) *V. chamaedrys* (2.1) *Galium album* (2.1)	*T. sect. ruderalia* (3.9)	*Ranunculus repens* (8.1)	*R. repens* (3.3) *T. sect. ruderalia* (2)

SR – stocking rate, LU ha^{-1}; TM – twice yearly mowed; SM – spring mowing; AG – autumn grazing; TRG – twice yearly rotational grazing; CG – continuous grazing; N – number of species; G – grasses; L – legumes; F – forbs

Stand-typical plant community was not described for the sites until 2011, but some dominant plants were already obvious (*Table 1*).

The effects of grazing (combined with mowing in S2) and mowing were observed in separate sections of each site from 2011-2014 (*Fig. 1*). Grazing regimes differed among the experimental sites and, in S2 and S3, from year to year (*Table 1*). Mean SR was generally low (0.66 LU ha^{-1}), although in S1, which had the highest SR (0.92 LU ha^{-1}) and the content of available plant nutrients, a heavy cattle load for about three weeks each year considerably damaged the plant cover. Site S2, where grazing was combined with mowing in 2011 and 2014 and replaced with mowing in 2013, had the lowest mean SR at 0.34 LU ha^{-1}. Grazing in S3 was carried out throughout the season with a mean SR of 0.73 LU ha^{-1}.

Mowed areas of 60 m^2 (6x10 m) enclosed by wooden fences were established randomly in each site in spring 2011 to be representative of plant community. Within this area, four 1 m^2 plots were delineated to assess herbage dry matter (DM) yield, weight proportions of functional plant groups, and herbage nutrient content by probability sampling. In addition, a permanent 16 m^2 (4x4 m) plot was delineated for phytosociological monitoring.

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For monitoring DM yield and herbage properties associated with grazing in S1 and S3 and with the combination of grazing and mowing in S2, four moveable 1 m^3 wire cages were placed randomly on each site in spring prior to the grazing season. Twice yearly, in accordance with mowed areas, the cages were moved several metres and the biomass under the cage was cut. Prior to the cage shift, the above-ground biomass at the new placement area was cut to a similar height of the grazed area. A permanent 16 m^2 plot was also established in grazed areas.

Soil single samples were taken in accordance with herbage samples each year 2012-2014 from soil layers of grazed and mowed sections at 4-8 cm and 10-14 cm depth to analyse pH (KCl), available plant nutrients, and organic matter content (*Table 2*). Available plant nutrient levels extracted using Mehlich III solution were categorized according to Sáňka and Materna (2004) in S1 as good/satisfactory (Ca, Mg), very high/high (K), and good (P). In S2, nutrient levels were satisfactory/low (Ca), low (Mg), low/satisfactory (K), and good/satisfactory (P); and in S3 satisfactory (Ca, Mg), satisfactory/low (K), and very high (P). The organic matter content ranged from good to very high (3.81 % ± s.d. 1.79).

Table 2. *Mean soil pH, available soil nutrient content (P, K, Mg, Ca, N_{min}), organic carbon (C_{org}), and total nitrogen (N_{tot}) content in experimental sites under grazing and mowing from 2012-2014 (sampled simply twice per year at 4-8 and 10-14 cm)*

Site	pH (KCl)		P (mg kg^{-1})		K (mg kg^{-1})		Mg (mg kg^{-1})		Ca (mg kg^{-1})		N_{min} (mg kg^{-1})		C_{org} (%)		N_{tot} (%)	
	grazed	mowed	grazed	mowed	grazed	mowed	grazed	mowed	grazed	mowed	grazed	mowed	grazed	mowed	grazed	mowed
S1	5.57	5.04	65.0	54.3	562.8	350.3	142.0	89.7	1982	1538	23.0	19.9	3.39	3.04	0.46	0.38
S2	4.84	4.39	56.6	42.9	85.5	170.2	30.6	29.8	1172	791	10.3	19.9	2.83	2.67	0.28	0.27
S3	4.11	4.23	251.2	153.0	131.7	90.2	84.8	69.7	1269	1008	13.4	14.1	5.98	4.22	0.46	0.41
Mean	4.84	4.59	128.2	91.5	270.2	210.2	89.1	69.7	1474	1112	16.0	18.3	4.14	3.44	0.41	0.37

Herbage biomass sampling

The 1 m^2 plots in both management areas were cut in early June and late September to 5 cm above the soil surface. Herbage was weighed, and proportions (% by weight) of graminoids (*Poaceae, Cyperaceae*, and *Juncaceae* families), legumes, and other forbs, considered functional plant groups, were determined. Herbage was desiccated and dry matter (DM) content determined. Dry matter herbage yield in tons ha^{-1} was calculated for each cut and combined for an annual DM herbage yield. Dried herbage from all 1 m^2 plots was crushed, and 100 g samples were weighed for determination of N, P, K, Ca, and Mg content in g kg^{-1}. Herbage N content was determined by the Kjeldahl method (ISO 11261, 1995). To determine P, K, Ca, and Mg content, samples were digested by nitric and perchloric acid in an open system, and concentrations of the elements in solution were determined spectrophotometrically by continuous flow analyser (P) and atomic absorption spectrometer (K, Ca, and Mg). The tetany ratio [K:(Ca+Mg)] was determined from values of K, Ca, and Mg content converted into milliequivalents per kilogram (Kayser and Isselstein, 2005). Annual N, P, K, Ca, and Mg removal in kg ha^{-1} by harvested biomass was calculated by multiplying DM herbage yield by the content of

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each nutrient for each cut. Values obtained for both cuts were combined. To estimate the limitation of grassland growth by nutrients nutrient ratios (N:P, N:K, K:P) were calculated according to Koerselman and Mueleman (1996), Olde Ventering et al. (2003) and Güssewell (2004). N limitation was designated as N:P ≤ 10 and N:K < 2.1, NP co-limitation as N:P >10 and < 16 and K:P > 3.4, K (co-)limitation as N:K > 2.1 and K:P < 3.4 and P limitation as N:P > 16.

Phytocenological assessment

The percent cover of vascular plant species (nomenclature following Kubát et al., 2002) was estimated visually in May, July, and late September/early October in 2011-2014 in the permanent 4x4 m plots. The first phytocenological assessment was conducted prior to grazing. The number of vascular plant species, Shannon-Wiener index (SW index), forage value (FV), and the Ellenberg nutrient indicator values (EIV-N) were calculated. Classification of species followed Regal (1980) and Veselá et al. (2009). The Ellenberg N indicator value (Ellenberg et al., 1992) was calculated as the mean of indicator values weighted by extent of cover of each species.

Statistical analysis

To evaluate the effects of management, site characteristics, and management/site (M-S) interaction on agronomic factors and plant diversity, the within-subject ANOVA model (R package nlme; Pinheiro et al., 2016) was used. To take into account the effect of repetition (pseudo-replication), the random factor (sampling date) was nested in site and in experimental year (temporal change) within the ANOVA model. Backward selection based on marginality rules was used for exclusion of non-significant effects from the ANOVA models. Reduced models were compared to the previous models using the Akaike Information Criterion. Post hoc comparison of the differences among tested groups was based on pairwise t-tests with *p*-values adjusted by the Holm correction (Pinheiro et al., 2016). Normality of the data was tested by the Shapiro–Wilk test. Data with non-normal distribution were transformed either by logarithmic transformation or by rank transformation in which the function *rntransform* from R package GenABEL (GenABEL project developers, 2013) was used. Relationships among measured variables were assessed by Pearson's correlation coefficient. All statistical analyses were processed using R (Pinheiro et al., 2016).

Results

Herbage biomass parameters

Herbage DM yield in 2011-2014 was significantly affected by management (*Fig. 2, Table 3*). Mean herbage DM yield decreased by 1.31 tons ha^{-1} with mowing (*Table 4*). The highest mean herbage DM yield was found in S1 (6.97 tons ha^{-1}), associated with satisfactory to very high available soil nutrient content (*Table 2*), which produced similar herbage DM yields under both managements in 2011-2013 (mowed 7.02, grazed 6.98 tons ha^{-1}). However, in 2014, the herbage DM yield in S1 dropped significantly in mowed plots (4.90 tons ha^{-1}) compared to grazed (8.89 tons ha^{-1}). In S2 and S3, mowing decreased mean herbage DM yield from 5.68 to 4.32 tons ha^{-1} and from 7.41 to 5.88 tons ha^{-1}, respectively.

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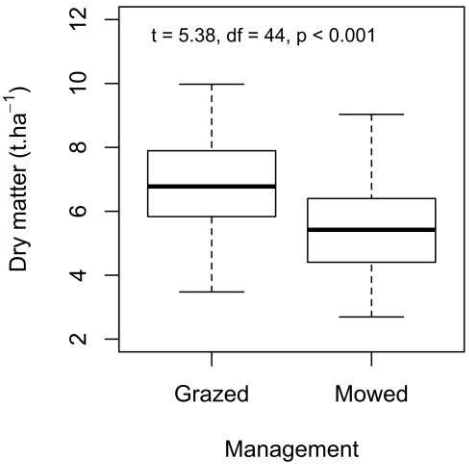

Figure 2. *Herbage dry matter yield of all sites associated with management*

Table 3. *The effect of predictors on studied parameters expressed by F-ratios in within-subject ANOVA model*

Parameters \ Predictors	Management	Site	M–S interaction	Transformation
Proportion of graminoids	25.43^{***}	17.92^{***}	9.45^{***}	rank(x)
Proportion of legumes	0.01 n.s.	20.89^{***}	43.62^{***}	rank(x)
Proportion of forbs	17.82^{***}	7.65^{**}	4.88^{**}	ln(x+1)
Herbage DM yield	28.98^{***}	Excl.	Excl.	Including 2011
Herbage N content	Excl.	62.20^{***}	Excl.	
Herbage P content	11.46^{**}	4.81^{*}	Excl.	
Herbage K content	43.99^{***}	194.59^{***}	Excl.	
Herbage Ca content	21.76^{***}	3.58^{+}	20.99^{***}	1/ln(x)
Herbage Mg content	4.71^{*}	104.65^{***}	Excl.	ln(ln(x))
Tetany ratio	79.78^{***}	28.26^{***}	5.16^{*}	ln(x)
Herbage N removal	46.07^{***}	Excl.	Excl.	ln(x)
Herbage P removal	40.24^{***}	Excl.	Excl.	
Herbage K removal	50.56^{***}	26.29^{***}	Excl.	ln(x)
Herbage Ca removal	1.64 n.s.	11.78^{**}	15.85^{***}	ln(x)
Herbage Mg removal	44.28^{***}	14.68^{***}	Excl.	ln(x)
Forage value	Excl.	4.34^{*}	Excl.	
Species richness	45.23^{***}	50.24^{***}	4.97^{*}	
SW index	8.93^{**}	19.91^{***}	Excl.	
Ellenberg N	5.52^{*}	102.92^{***}	Excl.	

***P < 0.001, **P < 0.01, *P < 0.05, + P < 0.1, n.s. – not significant, Excl. – excluded, M – Management, S – Site

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Table 4. *Mean management values in S1-S3 sites in proportion of functional groups (%), herbage DM yield (t ha^{-1}), herbage nutrient content (g kg^{-1}), tetany ratio, nutrient herbage removal (kg ha^{-1}), forage value, species richness, Shannon-Wiener index, and Ellenberg N indicator value*

Management	Site	Proportion of graminoids*	Proportion of legumes*	Proportion of forbs*	Herbage DM yield+	Herbage N content*	Herbage content*	Herbage K content*	Herbage Ca content*	Herbage Mg content*	Tetany ratio*
Grazed	S1	88.1	2.12	9.8	7.49	19.3	3.55	34.4	5.53	1.56	2.21
	S2	84.8	2.73	12.5	5.68	19.0	3.38	25.4	7.95	1.70	1.33
	S3	91.4	1.58	7.0	7.41	21.3	3.56	22.5	5.53	2.37	1.26
	All sites	88.4	2.1	9.8	6.86	19.86	3.50	27.43	6.33	1.93	1.60
Mowed	S1	77.7	0.62	21.7	6.46	17.8	3.20	29.0	8.56	1.68	1.48
	S2	65.4	17.4	17.3	4.32	19.5	3.30	22.4	11.55	1.69	0.88
	S3	91.6	0.32	8.1	5.88	20.3	3.28	17.9	5.47	2.43	1.04
	All sites	78.2	6.1	15.7	5.55	19.24	3.26	23.12	8.53	1.88	1.13
		Herbage N removal*	Herbage P removal*	Herbage K removal*	Herbage Ca removal*	Herbage Mg removal*	Forage value+	Species richness per 16 m^{2+}	Shannon-Wiener index+	Ellenberg N indicator value+	
Grazed	S1	132.0	24.5	247.5	37.1	11.4	73.6	15.3	1.74	6.42	
	S2	104.2	18.4	144.1	40.4	9.19	63.2	23.4	2.25	3.97	
	S3	153.0	25.6	163.6	38.4	17.3	64.7	18.6	1.83	4.39	
	All sites	129.7	22.8	185.1	38.6	12.6	67.2	19.1	1.94	4.92	
Mowed	S1	98.3	18.4	176.3	44.6	8.7	71.0	20.8	2.14	5.91	
	S2	75.1	12.8	89.2	42.1	6.5	67.0	26.4	2.48	3.71	
	S3	110.5	18.0	98.7	27.0	12.6	69.1	20.3	1.84	4.25	
	All sites	94.6	16.4	121.4	37.9	9.3	69.1	22.5	2.15	4.62	

*Values from 2012-2014, +values from 2011-2014

Proportion of functional groups was significantly affected by management for graminoids and forbs and by site and M-S interaction for all groups (*Fig. 3*, *Table 3*). With mowing, the proportion of graminoids decreased in S1 and S2 accompanied by increase in forbs (in both sites) and legumes (in S2), while legumes decreased in S1 and S3 (*Table 4*). The increase of high Ca demanding forbs was significantly correlated to decreased soil Ca availability in S1 (r = -0.62, p < 0.05, F= 6.36, df = 11). The increase of legumes in S2 was positively correlated with soil K availability (r = 0.72, p < 0.05, F = 6.49, df = 7). The decrease in legumes (*Trifolium repens*) in S1 and S3 was connected with soil Mg depletion (r = 0.42, p < 0.05, F = 4.78, df = 23).

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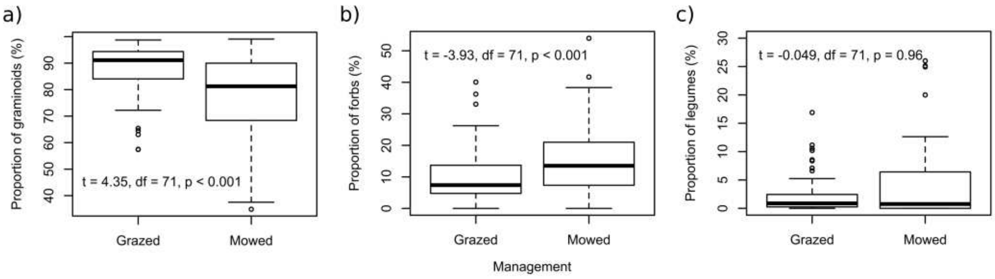

Figure 3. *Proportions of graminoids (a), forbs (b), and legumes (c) associated with management*

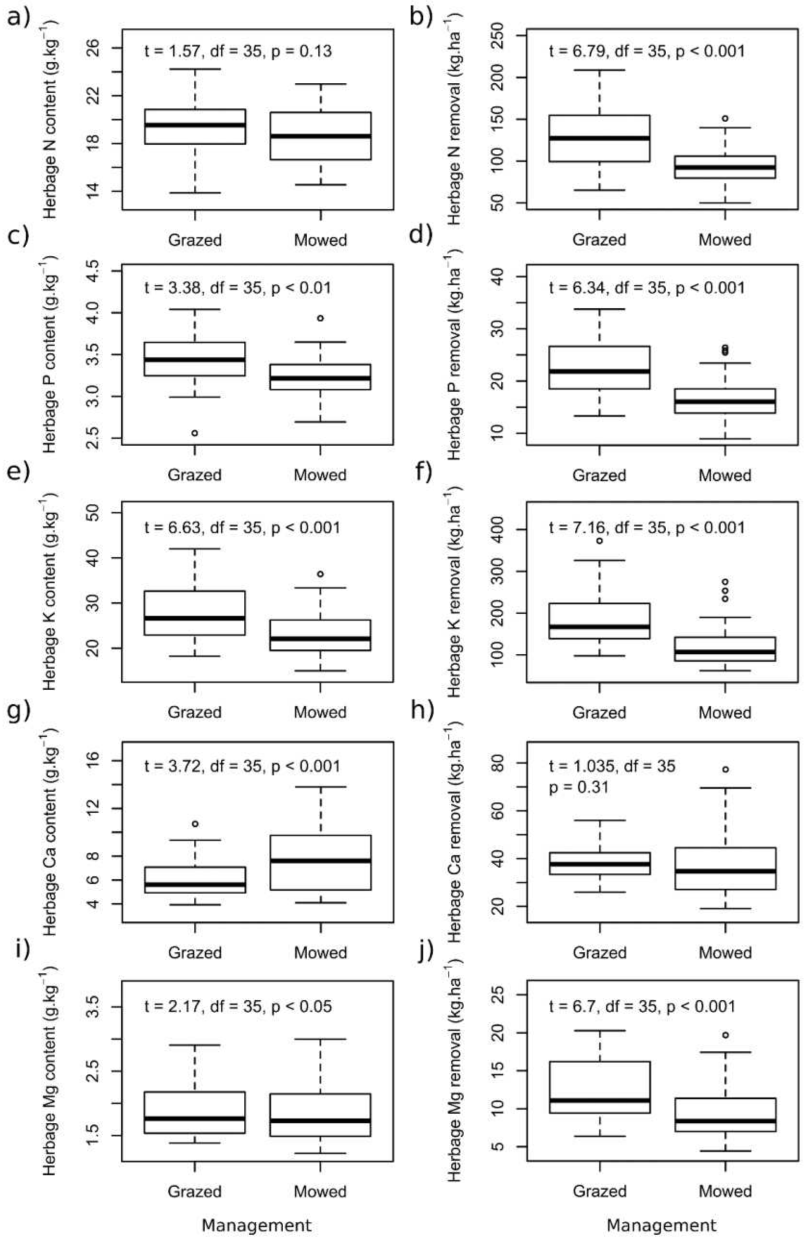

Figure 4. *The herbage N (a, b), P (c, d), K (e, f), Ca (g, h), and Mg (i, j) content and removal associated with management*

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Herbage nutrient content was significantly affected by management (P, K, Mg, and Ca), site (all nutrients), and M-S interaction (Ca) (*Fig. 4*, *Table 3*). Mowing depleting available soil nutrients by herbage removal decreased herbage P, K, and Mg content (*Table 4*) and altered the pattern of functional plant groups' occurrence. Across all sites, the reduction in herbage K content with mowing correlated strongly with depletion of soil K availability ($r = 0.60$, $p < 0.001$, $F = 16.87$, $df = 31$). Conversely, the herbage Ca content increased with mowing, which was significantly correlated with decrease in herbage DM yield (*Fig. 5*) and the proportion of graminoids (r = -0.78, $p < 0.001$, $F = 214.4$, $df = 143$) and with increase in forbs in S1 and S2 ($r = 0.61$, $p < 0.001$, $F = 27.12$, $df = 47$ and $r = 0.44$, $p < 0.01$, $F = 11.09$, $df = 47$) and legumes in S2 (r = 0.65, $p < 0.001$, $F = 34.11$, $df = 47$).

Herbage N, P, K, Mg, and Ca removal, which reflected the combined effect of herbage DM yield and nutrient content, was significantly affected by management (N, P, K, Mg), site (K, Mg, Ca), and M-S interaction (Ca) (*Fig. 4*). With mowing, the herbage N, P, K and Mg removal decreased (*Table 4*), while the herbage Ca removal increased markedly in S1, due to the increase in forbs proportion.

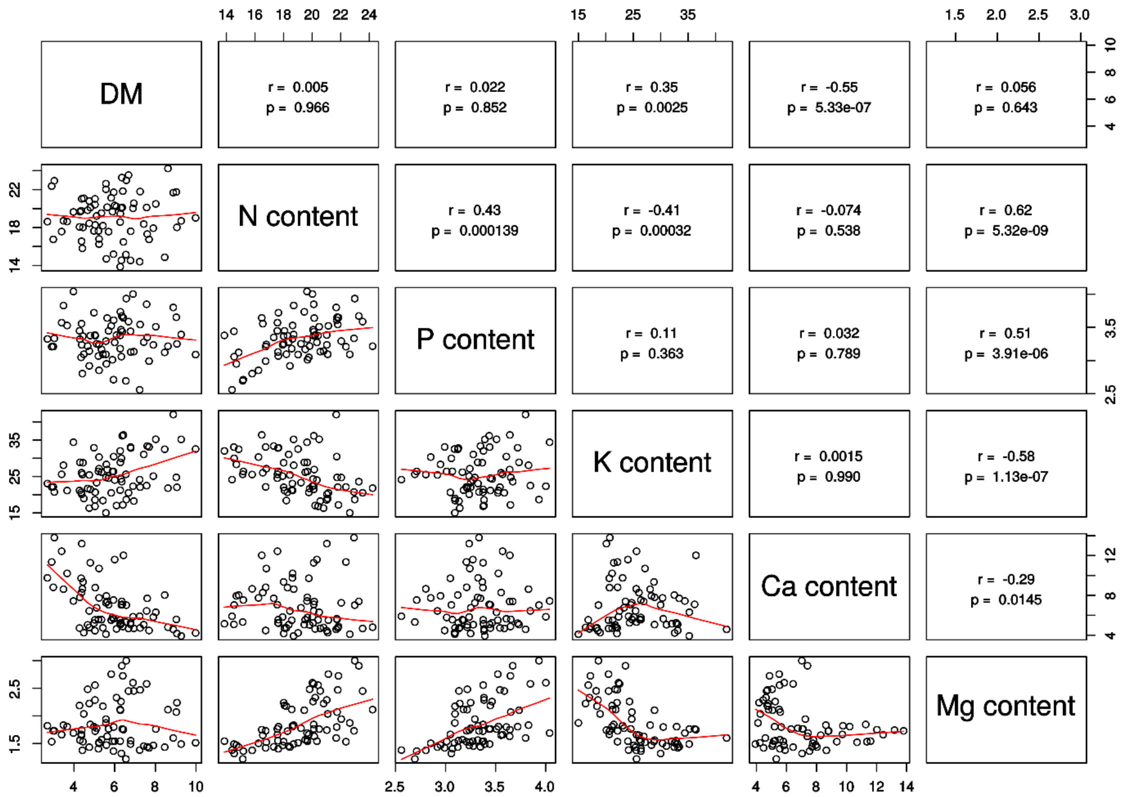

Figure 5. *Scatter plots of herbage dry matter yield (DM, tons ha^{-1}) and herbage nutrient content (g kg^{-1}) with correlation among variables. r is Pearson correlation coefficient, p is significance level. Data were fitted by spline model in the scatter plots*

Figure 5 shows significant relationships among herbage nutrients. The negative relationship between K and Mg content reflected the herbage K/Mg antagonistic relationship. The relationship between herbage N and K content in S2 was explained by significant spread of legumes with mowing, which increased the herbage N content but

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decreased the herbage K content. The mean herbage N content of < 20-22 g kg^{-1} in all sites was considered deficient for herbage production. The lowest herbage N content, seen in S1 under both managements, was negatively correlated with the herbage DM yield (r = -0.53, p < 0.001, F = 18.20, df = 47) induced by the dilution effect, i.e., a decrease in herbage nutrient content with the progressive accumulation of above-ground biomass. Similarly, nutrient ratios showed plant growth N limitation indicated by N:P ≤ 10 and N:K < 2.1 in all sites and both managements. Other type of nutrient limitations did not occur.

Forage value was significantly affected by site. The highest FV was observed in nutrient rich S1. Mowing in all sites was associated with a steady FV decline throughout the study period by approximately 10 points.

The tetany ratio was significantly affected by management, site, and M-S interaction (*Fig. 6, Table 3*). The tetany ratio was significantly declined with mowing (*Fig. 6, Table 4*). The highest tetany ratio in S1 (in grazed parts > 2.2) was caused primarily by high herbage K content. Lower tetany ratio, observed in S2 and S3, resulted from a mixed effect of lower herbage K content and higher herbage Ca content (S2) or Mg content (S3). The increase of tetany ratio coincided positively with herbage dry matter yield (r = 0.63, p < 0.001, F = 91.09, df = 143).

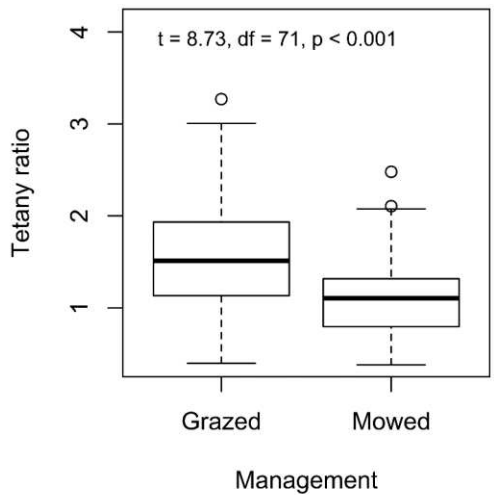

Figure 6. *Tetany ratio associated with management*

Species richness, Shannon-Wiener index, and Ellenberg N indicator values

Over years and across sites, mowing increased species richness and SW index compared to grazing (*Tables 3, 4, Fig. 7*). Both species richness and the SW index were substantially higher in S2 compared to S1 and S3 (mean of both managements), which may be attributed to lower cattle SR and available soil nutrients, combined with mowing in S2. In S1 and S2, mowing was associated with significantly higher species richness compared to the mowed section in S3.

The significant effect of management and site on EIV-N was revealed (*Fig. 8*). Nutrient removal by mowing reduced EIV-N (*Table 4*, *Fig. 8*). The highest EIV-N, seen in S1, were probably linked with the highest SR and soil nutrient levels. Nitrophilous species proliferated considerably in S1 (*Lolium perenne, Anthriscus sylvestris, Heracleum sphondylium*) compared to the more valuable forage species (*Lathyrus*

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pratensis, Vicia sativa, Lotus corniculatus, Tragopogon pratensis) found in S2. Conversely, the lowest EIV-N, showing the steepest decline throughout the trial, was recorded in S2 with medium nutrient availability and low SR combined with mowing. The Ellenberg N values were correlated negatively with species richness ($r = -0.49$, $p < 0.000$, $F = 22.13$, $df = 71$).

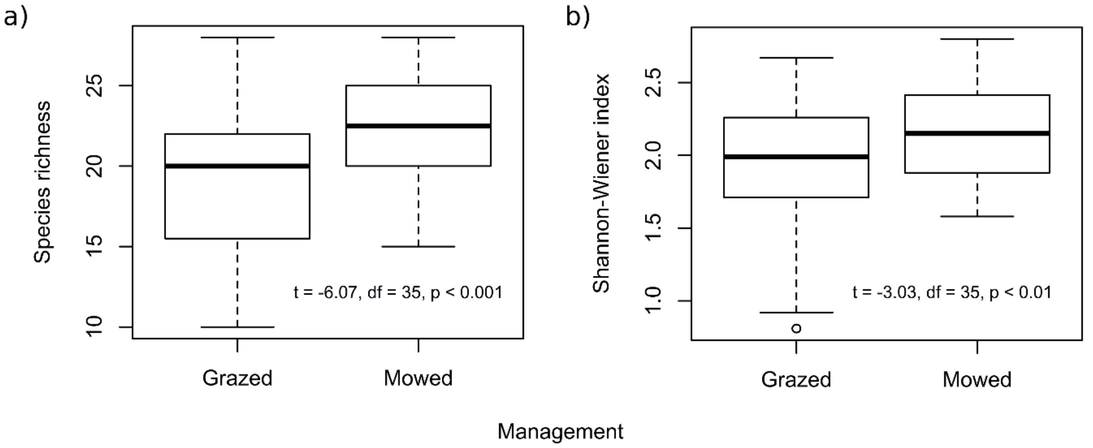

Figure 7. *Species richness per 16 m^2 (a) and Shannon-Wiener index (b) associated with management*

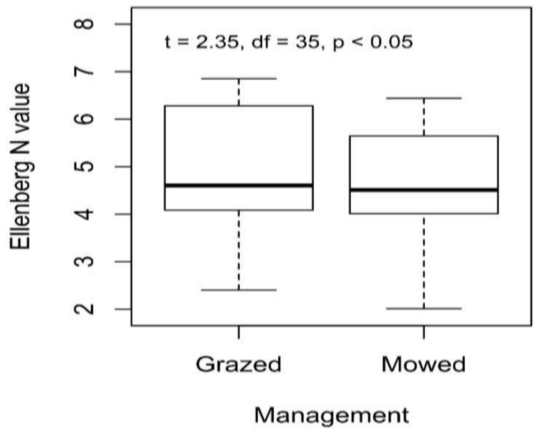

Figure 8. *Ellenberg N values associated with management*

Parameters obtained from the first phytocenological assessment (species richness, SW index, FV, EIV-N) were correlated with plant functional composition, herbage DM yields and nutrient contents of the first biomass sampling. Proportion of graminoids correlated negatively with species richness (r= -0.55**, *df* = 17) and S-W index (-0.72***, *df* = 17). Similarly, herbage DM yield was linked negatively with species richness (-0.54*, *df* = 23). Conversely, both species richness and SW index correlated positively with herbage Ca content (0.58**, *df* = 17 and 0.66**, *df* = 17, respectively) and S-W index reflected the increase in proportion of legumes (0.81***, *df* = 17). Forage value increased along with herbage DM yield (0.63***, *df* = 23), but declined due to increase in herbage Ca content (-0.60**, *df* = 17).

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Discussion

Our results summarize that short-term grazing exclusion with mowing can increase biodiversity parameters together with maintenance of agronomic demands in sites with different soil nutrient availability (including nutrient rich S1) and grazing types (twice yearly rotational grazing in S1 and mixed grazing types combined with mowing in S2). The only restriction, which prevented from biodiversity improvement via mowing, was high soil P availability supporting markedly spreading of competitive graminoids (S3). The increase of species richness linked with mowing was induced by changes in plant functional composition (i.e. by a decrease in cover of graminoids and an increase in cover of legumes and forbs) accompanied by acceptable herbage productivity and forage values.

Parameters of agronomic demands

High soil available P in S3 was responsible for grass species spreading in both mowed and grazed sections at the expense of forbs and legumes (Marriott et al., 2005; Hejcman et al., 2007). In contrast, in S1, and especially in S2, reduced P availability supported the decrease of graminoids related to mowing, which enabled forbs to regenerate to a greater extent than seen with grazing (Hejcman et al., 2007; Blüthgen et al., 2012). Cover of legumes were suppressed by mowing in high yielding S1 and S3, not only by decline in Mg availability, but likely – except climbing *L. pratensis* – by lack of light and defoliation (Marriot et al., 2009). The substantial increase of legumes in the exclusively mowed section of S2 correlating with soil K availability could be favoured by better light conditions (Kayser and Isselstein, 2005; Gilhaus et al., 2014).

Despite soil nutrient removal by mowing, water and nutrient availability was sufficient to keep herbage DM yields above 4 tons ha^{-1}; hence, the grassland types observed in the experiment could be considered highly productive (Hrevušová et al., 2015). Grazing provided higher herbage DM yields, even though lower yields of pastures than of meadows are usually reported due to livestock trampling and more frequent defoliation leading to photosynthesis restriction (Whitehead, 2000; Mládková et al., 2015). Even if 60-99% of nutrients ingested by livestock are returned to pasture in urine and manure (Jones and Tracy, 2014), distinct ratio of nutrients in excreta (P removal in animal body) than in forage decreases their effective utilization. Our opposite results could be related to quite low SR not leading to frequent trampling and defoliation and also to the type of methodology.

The highest yield, in grazed S1, was related to high nutrient availability (con N), maintaining almost the same herbage DM yields during the first three years after grazing was replaced by mowing. In S2 and S3, in which available nutrient levels were lower due to lower SR, herbage DM yield reduction with mowing was more pronounced. The observed inter-annual variability in herbage production is a common feature of all grassland types in Central Europe, primarily related to water supply (Pavlů et al., 2011).

Not only mowing, which decreased the proportion of graminoids, but also the type of grazing management affected the abundance of grasses. Tall grasses sensitive to frequent defoliation were in accordance with Pavlů et al. (2003) more abundant in rotationally grazed S1 compared to S3, where short grasses prevailed under continuous grazing.

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No significant differences in the herbage N content were observed between managements. This could be explained by the substantial increase of legumes in the mowed section of S2, which increased herbage N content due to N_2-fixing bacteria (Rose et al., 2012; Klaus et al., 2013b), while the herbage N content decreased with mowing in S1 and S3 with negligible proportion of legumes (Jones and Tracy, 2014). Nitrogen limitation of herbage production was indicated not only by the herbage N content, but also by the N:P (3.8-7.5) and N:K ratios (0.37-1.51) (Koerselman and Mueleman, 1996; Güssewell, 2004). In addition, in half the samples, mainly from S1 and S2, N was below the optimal range for dairy cattle of 19.2-25.6 g kg^{-1} (Whitehead, 2000). Nitrogen deficiency is common in pasture biomass (Mackay et al., 1995; Klaus et al., 2013a) and regulates other nutrient acquisition and translocation, such as P (Duru and Ducrocq, 1996). Hence, the positive relationship between the herbage N and P content may be explained by N deficiency.

Herbage P, K, and Mg contents significantly decreased with mowing, but P and K contents remained relatively high without any herbage P and K limitations. The herbage P content was considered optimal for dairy cattle, being 2.3-3.7 g kg^{-1} (Whitehead, 2000). In S3, herbage P content did not respond to extremely high soil P availability, further evidence of P uptake limited by N deficiency (Mengel and Kirkby, 1987).

Potassium uptake by plants under high K availability tends to exceed the threshold of physiological need of 28.0 g kg^{-1} (Hejduk, 2011) as shown in both managements of S1. High K availability supported by urine and manure deposition could result in inadequate uptake of Ca and Mg, impaired forage quality, and animal health problems related to grass tetany, the incidence of which is related to high plant growth rate (Kayser and Isselstein, 2005; Schonewille, 2013; Jones and Tracy, 2014). The risk of grass tetany (tetany ratio > 2.2) was almost completely eliminated in our study; only grazed parts in S1 with very high soil K availability revealed tetany ratio slightly over threshold value (2.21).

Herbage Ca content increased with mowing via forbs and legumes spreading (Reid, 1983). Contrarily, low herbage Ca content was linked with a predominance of grasses that are generally Ca and Mg poor in comparison to forbs and legumes (Daccord et al., 2001). Grass magnesium absorption is also affected by species. Powell et al. (1978) reported significantly greater Mg absorption in *Dactylis glomerata* than in *L. perenne*.

Along with the decrease in herbage nutrient contents and DM yields seen with mowing, herbage N, P, K, and Mg removal was reduced significantly (Kayser and Isselstein, 2005). Herbage Ca removal increase slightly with mowing in S1 and S2, due to the herbage Ca content increase (Reid, 1983).

Forage values ranged from 50 to 75 and were considered good (Grozavu et al., 2010), maintained by the grasses *Agrostis capillaris, D. glomerata*, *Poa pratensis,* and *L. perenne* in both managements and *Festuca rubra* in mowed areas, as well as the forbs *Taraxacum* sect. *Ruderalia* in grazed areas and *Plantago lanceolata, Achillea millefolium,* and *Pimpinella major* in mowed areas, along with legumes *T. repens* in grazed areas and *V. sativa* and *T. pratense* in mowed areas, all of which are attractive to animals due to medicinal and aromatic characteristics (Mládek et al., 2006). Legumes also are characterized by high protein and mineral contents. Similar to results of Hakrová et al. (2015), FV was found to decrease with increase in forbs. On the other hand, forbs improve livestock health, immunity, and forage consumption via aromatic substances (Mládek et al., 2006).

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Plant diversity

In agreement with Blüthgen et al. (2012) and Klaus et al. (2013a), land use and management were recognized as the major drivers of changes in plant diversity. Management regime is closely associated with herbage productivity, which is largely negatively related to plant species richness, often inter-correlated with herbage nutrient contents. In agreement with that, we recognized that plant species richness increased via suppressed competitive ability of grasses and spread of less productive forbs in mowed parts in S1 and S2, which was associated with the herbage Ca content increase (Duffková and Libichová, 2013; Duffková et al., 2015).

Due to more intense nutrient supply by pasturing before the study, species richness and SW index in mowed plots of S1 and S3 had lower values than grazed area of S2 with lower nutrient supply and occasional mowing (Marriot et al., 2009).

In addition, there are other important factors such as soil site properties (pH and available nutrients), which determine plant species richness. High soil P availability was designated as an important factor relating negatively to species richness (Hejcman et al., 2010; Merunková and Chytrý, 2012). Hence, the lack of increase in species numbers, along with the high proportion of graminoids under mowing in S3 may be explained by the extremely high P availability, which allowed highly productive species to dominate (Hejcman et al., 2007). Phosphorus persists in the soil unaffected for decades and thus, it is not worth trying short-term mowing as a tool for plant diversity improvement in such sites (Semelová et al., 2008).

High soil K availability in S1 provided sufficient K supply for forbs increase together with decreased productivity and better light conditions in mowed plots (Elberse et al., 1983).

In accordance with Briemle et al. (2002), the adaptation to more intensive grazing in S1 and S3 was pronounced for cover of low-growing rosette legume *T. repens,* which decreased in mowed parts under lower soil Mg content. Spread of nitrophilous species in S1 was observed as a consequence of soil nutrient supply by cattle excrements (Plantureux et al., 2005).

The highest EIV-N (S1) were associated with broad nutrient supply (Blüthgen et al., 2012) and endozoochorous dispersal (Albert et al., 2015). Conversely, the lowest EIV-N (S2) in the exclusively mowed section reflected previously low SR.

Conclusions

A four-year experiment conducted in upland pasture found herbage DM yield, herbage nutrient contents (K, P) and nutrient removal (N, P, K, Mg) to be decreased by mowing twice a year replacing low intensity cattle grazing, but these agronomic factors remained acceptable for dairy cattle nutrition. Although our set of data was limited, such clear benefits as wider plant diversity related to legume and forb spreading and the elimination of the risk of grass tetany (i.e. tetany ratio < 2.2) with mowing were associated with a herbage K decrease and increase in herbage Ca content. To reconcile biodiversity and agricultural targets on pastures, a compromise between maintaining grassland species richness and herbage properties according to site-specific conditions may be required. Our findings on short-term grassland management changes are relevant to farmers' priorities when they operate within sustainable agro-environmental schemes.

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DOES THE ALTITUDE AFFECT THE STABILITY OF MONTANE FORESTS? A STUDY IN THE KAHUZI-BIEGA NATIONAL PARK (DEMOCRATIC REPUBLIC OF THE CONGO)

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Abstract. To understand the functioning of montane forests, this study was conducted in the highlands of the Kahuzi-Biega National Park in the Democratic Republic of the Congo. The relationship between the altitude and the floristic stability of woody layers and regeneration capability of canopy species after many years of disturbance was studied. Ten 1-ha plots were established from 1935m to 2760m a.s.l. In each plot we inventoried the trees ≥10cm of diameter at breast height (DBH), separating a canopy layer (10% of the tallest trees) and an understorey layer (all the other trees). In each plot, we nested a 0.1 ha subplot to inventory the saplings between 1 and 10 cm DBH. We found that the Jaccard index of dissimilarity between the understorey layer and the canopy layer decreases with the altitude. The proportion of species which are well represented in the three layers increases with the altitude. The number of pioneer species decreases with the altitude while that of non-pioneer and shade tolerant species increases. These findings suggest that altitude influences the stability of highland forests, higher altitude being more stable than lower ones in the case of this study.
Keywords: *highland forests, regeneration capability, woody layers, Jaccard index of dissimilarity, non-pioneer species*

Introduction

Montane forests are biodiversity-hotspots and are among the most species-rich ecosystems worldwide (Bussmann, 2001a, 2001b). They are also particularly rich in uncommon and endemic species (Plumptre et al., 2006; Skarbek, 2008). Their high diversity is due to a great variety of environmental conditions (Clinebell et al., 1995; Richter et al., 2009) as well as persistence of favorable climatic conditions during past geological times, which has allowed the survival of numerous plant species (Dimitrov et al., 2012). They are nevertheless one of the most fragile ecosystems (Sharma et al., 2009) and one of the most vulnerable to global change (Lenoir, 2009). Their

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vulnerability is due to the fact that they cover rather small areas at the Earth's scale and that they are governed by specific environmental conditions (geomorphological factors, soil, cloudiness, humidity, temperature,…) which may rapidly change under climate warming and human pressure (Fjeldså and Lovett, 1997; Körner, 2009; Skarbek, 2008; Nzigidahera, 2012).

In tropical forests, numerous studies on montane forests have focused on the relationship between altitude and structure, or between altitude and floristic composition (Hedberg, 1951; Jamir et al., 2006; Hemp, 2006). Several studies have shown that lower altitudes are home to taller trees than higher altitudes (Ashton and Hall, 1992; Siebert, 2005; Richter et al., 2009), even in small elevation gradients (Gonmadje et al., 2017). In the same way the lower altitude forests differ from the higher ones in their floristic composition (Schmitt et al., 2010; Gonmadje, 2012; Admassu et al., 2016; Imani et al., 2016), with a decrease of species diversity with increasing altitude (Doumenge, 1998; Lomolino, 2001; Tassin et al., 2004; Hemp, 2006; Mangambu et al., 2013; Mwanga Mwanga et al., 2014; Admassu et al., 2016; Imani et al., 2016), even if not systematically (Siebert, 2005; Delnatte, 2010; McCain and Grytnes, 2010; Salas-Morales and Meave, 2012).

Some studies in the montane forests focused on their dynamic patterns through space and time (Fine et al., 2005; Zhang and Zhang, 2007; Njunge and Mugo, 2011). However, the study of vegetation dynamics requires observations through relatively long time scales. When such observations are unavailable, a comparison of the floristic composition between forest layers – canopy, understorey, sapling layers - can lead to some interesting results on the possible dynamics between these layers (Kalacska et al., 2004; Schulze et al., 2005; Behera and Misra, 2006). This approach is simple when it is used for forests dominated by one species and becomes more complex in forests with many species and a diversified canopy (Woods and Whittaker, 1981). Taking into account both the abundance and the occurrence of species in the considered forest layers can spread some light on the vegetation dynamics (Hall and Swaine, 1976; McEwan et al., 2005; Amani and Lejoly, 2014). Such studies were performed in various lowland forests (Letouzey, 1957; Woods and Whittaker, 1981; Swaine and Hall, 1988; Connell and Lowman, 1989) but in a limited number of highland forests (Njunge and Mugo, 2011; Shiels and Walker, 2013).

Based on the abundance patterns of species within different layers, Pivellon and Coutinho (1996) distinguished "stationary" stable forests and "transient" unstable forests or changing forests. Stable forests are those in which floristic composition of the canopy layer does not differ significantly from that of the lower forest layers. Connell and Lowman (1989) also defined stable forests as those characterized by one or more similar dominant species in the canopy and lower layers (meaning that these species will likely dominate the canopy in next generations) whereas unstable forests are considered to be characterized by one or more species which are currently dominant in the canopy but become scarce in lower layers. From a floristic dynamics point of view, it is expected that tree species characterizing the current canopy will die and eventually be replaced by the species found in the current sapling and understorey layers which are able to reach the canopy (Hallé et al., 1978; Woods and Whittaker, 1981; Nicotra et al., 1999).

It is generally agreed that young forests tend to have more canopy species with few – if any – regeneration in the sapling and understorey layers while old growth and stable forests are mainly dominated by canopy species with abundant regeneration in the sapling and understorey layers. In other words, forests which have experienced some disturbance

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are expected to incorporate in their canopy a higher proportion of poorly regenerating pioneer species than old growth forests (Connell and Lowman, 1989; Pivellon and Coutinho, 1996; Vandermeer and Granzow de la Cerda, 2004; Yang et al., 2008).

In a case of non-disturbance or equal disturbance between lower and higher altitudes, lower altitudes develop more stable forests than higher ones. Due to the sensibility of higher altitudes to global change, they are liable for instability (Pierlot, 1966; Njunge and Mugo, 2011). These forests thus tend to be dominated by many pioneer species (e.g. forests at higher altitudes in general, Pierlot, 1966) and could be more instable (Wood and Whittaker, 1981) compared to forests at lower altitudes, but this general trend is reversed in a case of unequal disturbance history between lower and higher altitudes (Runge, 2007).

The forest ecosystems in the highlands of the Kahuzi Biega National Park (KBNP) are spanning from 1800 to 3315 m of altitude. Some of them have been severely disturbed as a consequence of recurrent political turmoil (and its subsequent wars and various armed conflicts) in the Democratic Republic of the Congo (DRC) and neighboring countries (World Heritage, 2005; Jepson et al., 2015). Though some studies reported the consequences of these anthropogenic pressures on the vegetation of the KBNP (Masumbuko, 2011; Kabonyi et al., 2011), very few is known regarding the regeneration and restoration capabilities of its forest ecosystems, more particularly in its highland areas. The role of altitude in the restoration processes has also been poorly studied (Gonzalez et al., 2013; Njunge and Mugo, 2011) while the lack of such information for the KBNP highland's forests is detrimental to the evaluation of their sensitivity to global change and the development of a sustainable management strategy (Dupuy, 1998; Dupuy et al., 1998). In the KBNP, it can be assumed that these political conflicts disturbed more the lower altitudes than higher altitudes as it is easier to make camping or logging activities in lower altitudes than going far at mountain's summits in higher altitudes (Magrath et al., 2007)

Here we investigated the stability state of these highlands forests with respect to disturbance history and in relation with the altitude. We evaluated the dynamic trends of these montane forests by testing if 1) the dissimilarity between the canopy layer and understorey layer decreases with the altitude; 2) the most abundant canopy species remain abundant in lower layers at higher altitudes; 3) the proportion of pioneer species decreases with the altitude and that of non-pioneer species increases with the altitude in the three layers.

Material and methods

Study area

The study was conducted in the Kahuzi Biega National Park (KBNP) which is located in the eastern part of the DRC, between 28.45°E - 28.85°E of longitude and 2.66°S - 2.09°S of latitude. The KBNP harbours three main vegetation types according to the altitude, including lowland forests (stretching from 700 m to 1250 m a.s.l), submontane forests (from 1250 m to 1800 m) and highland forests from 1800 m to 3315 m a.s.l (Fischer, 1993; Mangambu, 2013). This study covers only the highland forests (*Fig. 1*).

The KBNP is established in two main climatic zones. The lowland areas undergoes an equatorial climate where it rains almost throughout the year. Precipitations are high and can reach more than 2 600 mm; temperature varies between 15 and 25°C. In the

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highland areas and submontane forests, the climate is characterized by 3 to 4 months of dry season and lower rainfall (mean: 1 900 mm; Fischer, 1993). The highlands are dominated by hills and marshes. They are located in the Albertine rift, a biodiversity hotspot (Plumptre et al., 2006, Poulsen et al., 2005). In terms of phytogeography, the highlands are part of the afromontane center of endemism but the lowlands belong to the guineo-congolese center of endemism (White, 1983).

The highlands of the KBNP are dominated by a vegetation which is structured in 3 main strata including arborescent strata, understorey strata and herbaceous strata. This forest has many epiphytes especially lichens, ferns and many *Orchidaceae* species. The arborescent strata is characterized by species such as *Albizia adianthifolia, Neoboutonia macrocalyx, Dombeya torrida, Macaranga capensis, Polyscias fulva, Alangium chinense, Shirakiopsis elliptica, Pleiocarpa pycnantha, Syzygium guineense, Symphonia globulifera, Tabernaemontana stapfiana, Carapa grandiflora, Nuxia floribunda, Lachnopylis thomensis, Agauria salicifolia, Morella salicifolia, Podocarpus milanjianus, Afrocarpus usambarensis*… The understorey strata is constituted by *Hagenia abyssinica, Maesa lanceolata, Galiniera saxifraga, Psychotria mahonnii, Rapanea melanophloeos, Cyathea manniana, Erica arborea, Alchornea hirtella, Chassalia subbochreata...* In the herbaceous strata we can see species like *Sinarundinaria alpina, Lobelia gibberoa, Salacia sp, Urera hypselodendron, Pilea sp, Ipomoea involucrata, Scadoxus multiflorus, Hypoestes trifolia, Sericostachys scandens, Centella asiatica, Ageratum conyzoides, Aspilia kotschyii, Helichrysum spp, Microglossa pyrifolia, Mikania spp, Senecio spp, Vernonia spp, Impatiens spp, Begonia spp, Cuscuta kilimanjari...* and many ferns.

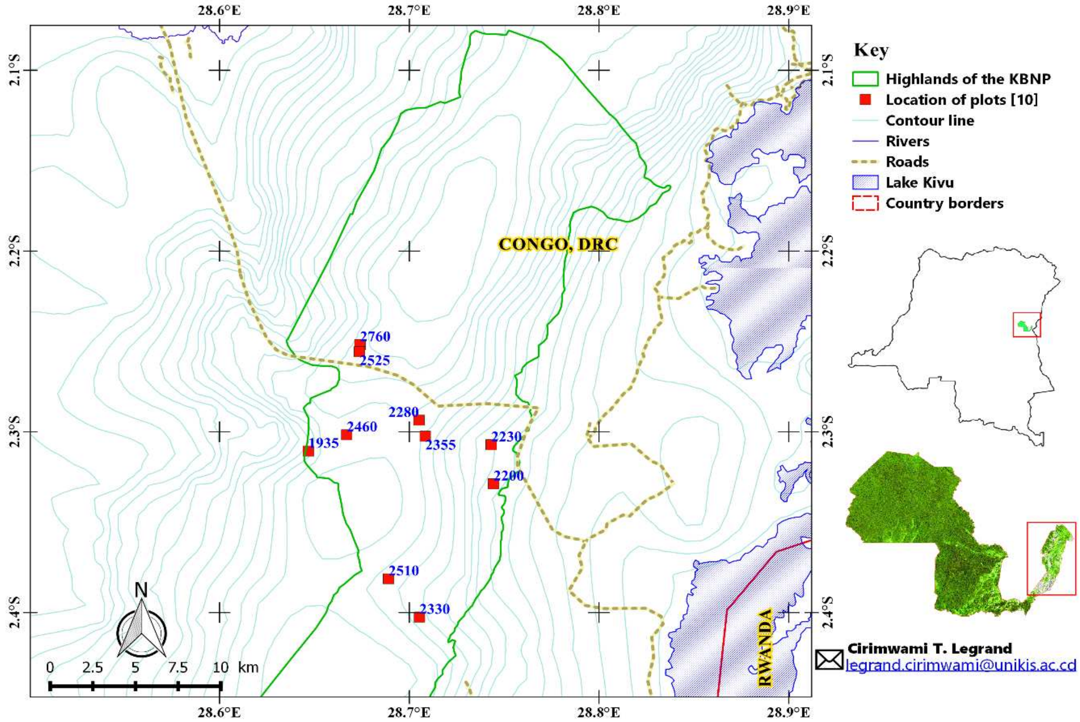

Figure 1. *Location of the ten studied plots in the highlands of the Kahuzi-Biega National Park in the Democratic Republic of the Congo.*

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Data collection

A total of 10 1-ha (200 m x 50 m) nested plots (displaying the canopy layer, the understorey layer and the sapling layer) were established between 1935 and 2760 m a.s.l. As there are many conflicting statements on the minimum height of canopy species in montane forests (Lebrun and Gilbert, 1954; De Barcellos Falkenberg et al., 1995), we arbitrary considered the 10 % tallest trees of the stand as belonging to the canopy layer, the remaining trees belonging to the understorey layer. Trees of the canopy and the understorey layers and reaching at least 10 cm of diameter at breast height (DBH), were inventoried within (1 ha) plots. For this category of trees, height and diameter were measured, respectively by a Laser Ace and a DBH-meter. The height varied from 2 to 28 m while the diameter varied from 10 to 155 cm. Inside each 1-ha plot, the sapling layer (comprising all woody species between 1 cm and 10 cm of DBH) was inventoried within 0.1 ha (200 m x 5 m) subplots. The altitude was recorded using an altimeter SUUNTO Vector (± 5 m) while the geographic coordinates were recorded with a Garmin GPS (± 3 m of precision).

Species identification

Most species were directly determined in the field. The remaining species were later identified at the Herbarium of the Lwiro research center, by comparing collected plant samples and using existing floras (Troupin and Bridson, 1982; Fischer and Killmann, 2008) and to the online flouring plant database of Geneva (African Plant Database, Lebrun and Stork, 1991-2015). We identified 87.6 % of all species till to the level of species, 9.3 % to the level of genera and 3.1 % of all species were not identified. We used the nomenclature proposed by the Angiosperm Phylogeny Group (APG IV) in 2016 (Chase et al., 2016).

Data analysis

Species richness and diversity indices

In each plot and for the canopy and understorey layers, species richness was estimated using the rarefaction index (r) while diversity was calculated by the Fisher alpha index (α). The rarefaction index (*Eq. 1*) for species richness estimations represents the expected number of species in a sample of n individuals selected at random from a collection containing N individuals, S species and N_i individuals in the i^{th} species (Hurlbert, 1971). If a random sample of n individuals is taken from each community, the number of species recorded increases with the number of individuals sampled (Bell, 2000), that is why it is better to analyse the species richness with standardized number of individuals within plots. This index is:

$$r = E(S_n) = \sum_1 \left[1 - \frac{\binom{N-N_1}{n}}{\binom{N}{n}} \right] \quad \text{(Eq. 1)}$$

The Fisher alpha index was calculated from the formula proposed by Fisher et al. (1943). This index is mostly influenced by rarer species (Magurran, 2004) and estimates the number S of species with N observed individuals (Fisher et al., 1943; Magurran, 2004), it is frequently used in forest related studies (Gonmadje et al., 2011, Gourlet-Fleury et al., 2013). We used this index as it considers the whole individuals sampled in

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each plot without any standardization of individuals as it is in the previous index, it calculates the diversity.

Variation of woody layers floristic dissimilarity along the altitudinal gradient

We compared the floristic compositions of the two layers (canopy and understorey) within the established 10 plots. The Jaccard index of dissimilarity was calculated between each pair of plots situated at the same altitude one belonging to the understorey and another to the canopy. This allowed to quantify the variation in floristic composition of communities at each altitude (Jaccard, 1908; Legendre et al., 2005; Tuomisto and Ruokolainen, 2006). We then made the correlation between this dissimilarity index and the altitude to assess the way the altitude and related factors can influence the floristic stability in this area).

Correlation between "well represented species" and the altitude

The distribution of canopy species into the canopy layer, understorey layer and sapling layer were performed. We calculated the relative abundance of each species in the three layers plot by plot and compared those three values using a Chi² test to test if those relative abundances show any statistical difference or not. If the difference is significant, the distribution of the considered species is not equilibrated in the three layers. If the difference is not significant, it means that the relative abundance of the species in the canopy is not different statistically from its relative abundance in the understorey and sapling. These species are considered as well represented species in the three layers. We then made a correlation of the proportion of well represented species and the altitude to test if higher altitudes have more "well represented species" than lower altitudes.

Light requirement of species in the three layers

The light requirement (LR) of species informs on the degree of disturbance in a forest (Hawthorne, 1996; Van Gemerden et al., 2003a). The LR of each species was found in the literature especially in Lebrun and Gilbert (1954), Pierlot (1966), Lovett et al. (2006) and in the database developed by Bénédet et al. (2016). Three categories were considered: Pioneer species, Non-Pioneer Light Demanding species and Shade tolerant species. Species for which we didn't find their LR were categorized as "Unclassified". In each layer we calculated the proportion (*Eq. 2*) of individuals belonging to each LR category:

$$PX_A = \frac{\text{Individuals of } X_A}{N_A} \times 100 \qquad \text{(Eq. 2)}$$

Where PX_A is the proportion of individuals belonging to X light requirement category present in the plot A and N_A the total number of individuals in the plot A. The number of individuals belonging to each LR were correlated with the altitude layer by layer.

For correlations, the Pearson method were used as our data required a parametric option. For regressions, we used linear models. All statistical analyses were performed with R software (R Development Core Team, 2015) using the vegan (Oksanen, 2017) and ecodist package (Goslee and Urban, 2007).

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Results

Richness, diversity, and their relationship with altitude

General characteristics of the stands

We inventoried a total of 7590 individuals: 1940 in the sapling layer (194 ± 58.6 per 0.1 ha subplot), 5087 in the understorey layer (508.7 ± 176.8 per 1 ha plot) and 563 in the canopy layer (56.3 ± 19.8 per 1 ha plot). A total of 62 species (17.6 ± 3.75 per 0.1 ha subplot) belonging to 34 families were collected in the sapling layer, 82 species (22.7 ± 9.04 per 1 ha plot) belonging to 37 families in the understorey layer whereas in the canopy layer 45 species (10.8 ± 2 per 1 ha plot) belonging to 29 families were collected.

In term of number of individuals, the most abundant families are the *Rubiaceae* followed by the *Monimiaceae* in the sapling layer, the *Euphorbiaceae* followed by the *Rubiaceae* in the understorey layer and the *Euphorbiaceae* followed by the *Myrsinaceae* in the canopy layer. The richest families are the *Rubiaceae* (10 and 16 species) in the sapling and understorey layers and the *Euphorbiaceae* (5 species) in the canopy layer. One of the 62 species of the sapling layer was only identified at the family level (*Rubiaceae*) and was represented by 3 individuals at 2355 m a.s.l. Three of the 82 species of the understorey were only determined at the family level (*Rubiaceae*). The three species were represented by only one individual and were collected at 1935 m a.s.l.

The relationship between numbers of individuals, numbers of species, Fisher-Alpha index of diversity, rarefaction index of richness and altitude are represented below (*Fig. 2* and *Table 1*).

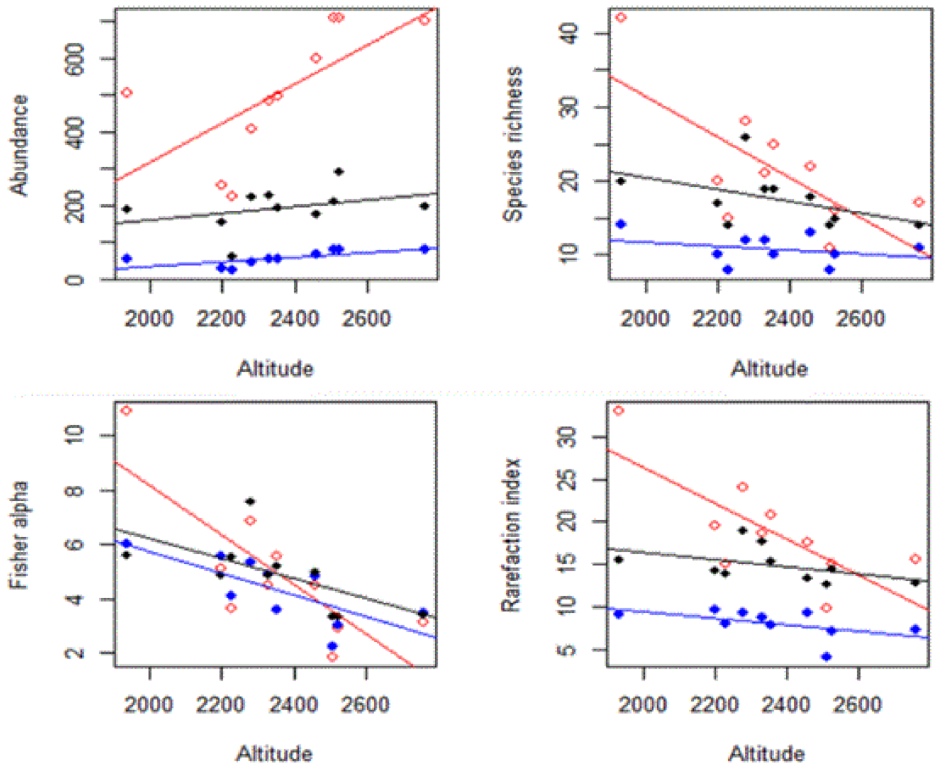

Figure 2. *Relationship between number of individuals, species richness, Fisher-Alpha index, Rarefaction index and altitude in the three layers. The sapling layer is represented by the black line, the understorey by the red line and the canopy by the blue line.*

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Within the three layers, the number of individuals increases with altitude, significantly for the understorey and the canopy (*Fig. 2*) while the species richness, the Fisher-Alpha index and the rarefaction index decrease with the altitude, but not significantly in most cases.

Table 1. *Correlations between number of individuals, number of species, index of rarefaction, index of Fisher-Alpha and altitude in the three layers. "r" is the coefficient of correlation of Pearson, R^2 the coefficient of determination and bold p-values show significant correlations at α = 0.05. The sapling layer was inventoried in 0.1 ha plots.*

Layer	Variables	r	R^2	p-value
UNDERSTOREY	Individuals/Altitude	0.67	0.45	**0.034**
	Species/Altitude	-0.71	0.5	**0.022**
	Rarefaction/Altitude	-0.75	0.56	**0.012**
	Fisher-Alpha/Altitude	-0.78	0.64	**0.006**
CANOPY	Individuals/Altitude	0.67	0.44	**0.035**
	Species/Altitude	-0.31	0.1	0.38
	Rarefaction/Altitude	-0.52	0.27	0.12
	Fisher-Alpha/Altitude	-0.73	0.53	**0.02**
SAPLING	Individuals/Altitude	0.34	0.11	0.34
	Species/Altitude	-0.47	0.22	0.17
	Rarefaction/Altitude	-0.45	0.2	0.19
	Fisher-Alpha/Altitude	-0.64	0.41	**0.048**

Relationship between floristic dissimilarity and altitude

The dissimilarity between the understorey and the canopy layer of each plot were calculated according to the altitude (*Fig. 3*), to study whether the floristic stability of this ecosystem is correlated to the altitude.

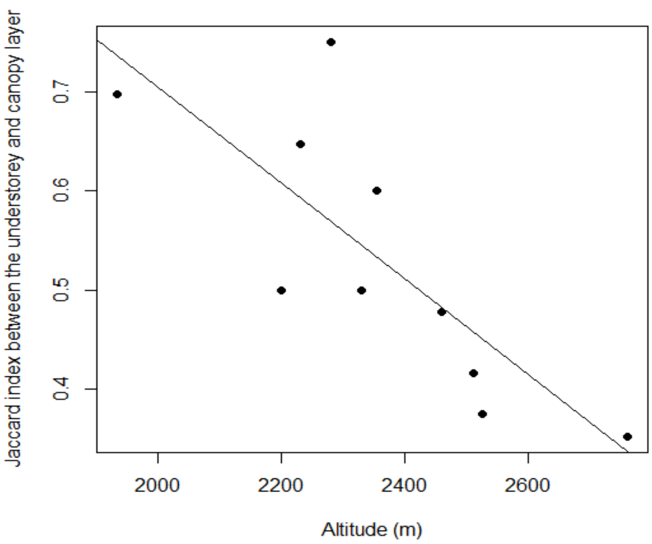

Figure 3. *Correlation between the floristic dissimilarity (Jaccard index) between the understorey and the canopy layers and the altitude.*

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The Jaccard index of dissimilarity significantly decreases with altitude (r = - 0.79, p = 0.006). This decrease confirms a more homogeneous floristic composition between the understorey and canopy layers when the altitude increases. Homogeneous floristic composition between the understorey and the canopy layer is a sign of floristic stability as this means that the species which are present in the canopy layer at higher altitude are also well represented in the understorey layer.

Floristic and functional composition of the three layers and their relationship with altitude

Distribution of the canopy species into the three layers

In the *Figure 4*, the percent of canopy species for which the proportion of their relative abundance (RA) in the three layers do not differ statically are presented. The relative abundances of canopy species in the canopy, the understorey and the sapling layers were computed and compared through a Chi² test (see appendix for more details).

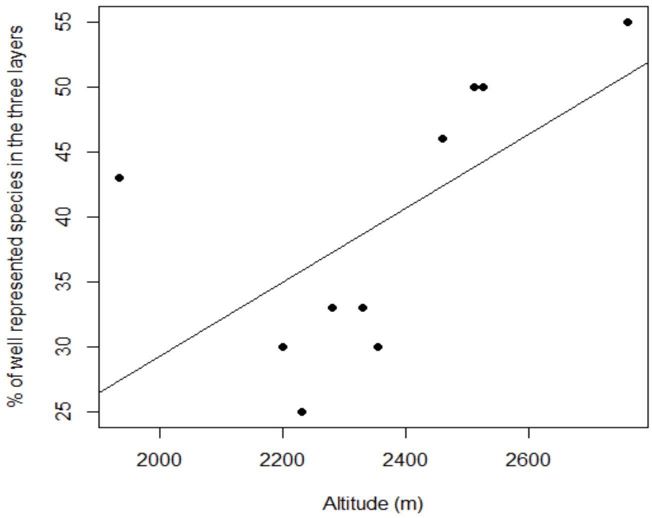

Figure 4. *Correlation between the percent of well represented species and the altitude. Well represented species are those for which their relative abundance in the three layers do not differ significantly.*

As shown in the *Figure 4*, the percent of well represented species in the three layers tends to increase with the altitude (r = 0.61, p-value = 0.06). This suggests that most abundant species in the canopy still in more cases abundant in lower layers particularly at higher altitude plots. The species which do not follow this trend are those which are abundant in the canopy but become scarce in lower layers or they are abundant in lower layers and become scarce in the canopy (see species with p-values ≤ 0.05 in the appendix).

Correlation between light requirement and altitude in the three layers

The variation of the light requirement (pioneer species, non-pioneer light demanding species: "NPLD" and shade tolerant species) is presented in the *Table 2* to test if they are correlated to the altitude in the three layers. The aim in this computation is to assess if the number of pioneer species is negatively correlated to the altitude and the number of non-pioneer light demanding and shade tolerant species are positively correlated to the altitude.

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Table 2. *Correlation between the number of individuals belonging to each light requirement (LR) category within the three layers and the altitude. Bold p-values represent significant correlations at α = 0.05. The proportion of each LR category is represented in the % column.*

	Sapling layer			Understorey layer			Canopy layer		
Light requirement	*%*	*r*	*p*	*%*	*r*	*p*	*%*	*r*	*p*
Pioneer species	6.3	-0.72	**0.02**	16.3	-0.7	**0.03**	21.8	-0.67	**0.03**
Non-pioneer light demanding species	26.0	0.65	**0.04**	43.9	0.61	0.06	64.0	0.85	**0.002**
Shade tolerant species	41.4	0.63	0.05	33.6	0.35	0.32	13.3	0.03	0.93
Unclassified species	26.3	-0.1	0.78	6.2	0.17	0.64	0.9	0.03	0.93

In terms of individuals, non-pioneer light demanding species are more represented in the whole studied area (40.8 %) followed by shade tolerant species (34.1 %), pioneer species (14.1 %) and unclassified species (11 %), the Chi squared test showed a significant difference ($\chi^2 = 25.59$, df = 3, $p < 0.0001$). The number of pioneer species generally decreases with the altitude this pattern being significant in the three layers. On contrary, the number of non-pioneer light demanding species and the number of shade tolerant species tend to increase with the altitude. This correlation is significant for the non-pioneer light demanding species in the sapling and the canopy layer.

Discussion

The species richness and diversity of woody layers decrease with altitude

In some montane forests, the species richness and diversity of woody plants begins to decrease above 1500 m a.s.l (Gentry, 1988). This decrease is due to multitude factors which vary with the altitude including the geomorphological factors, soil, humidity, cloudiness, temperature… (Delnatte, 2010; Malhi and Phillips, 2005). This pattern is also observed for non woody plants (Raman et al., 2005; Richter et al., 2009) even if the opposite trend (increasing species richness and diversity with altitude) can be observed, for example, in rattan communities in Indonesia (Siebert, 2005) and for the *Melastomataceae* family in French Guiana (Delnatte, 2010).

In the highland areas in the KBNP, species richness of plants and their diversity decreases with altitude for the *Rubiaceae* family (Mwanga Mwanga et al., 2014) and for ferns (Mangambu et al., 2013). We obtained the same result for the total woody floristic composition in the sapling, understorey and canopy layers. This confirms the findings of Imani et al. (2016) who found the same trend in a study conducted in the same ecosystem and in the surroundings of the Park in which they only considered tree species ≥10cm DBH. The higher altitudes are characterized by a lower number of species and a lower diversity. Nevertheless, the number of individuals increases with the altitude, the same pattern was found by Gonmadje et al. (2011) in Cameroonian montane forests and Imani et al. (2016) in the KBNP and its surroundings.

Forest stability in the Kahuzi-Biega National Park's highlands

The dissimilarity between the canopy and understorey layer decreases with altitude

The canopy layer plots at higher altitudes are more similar to their understorey layer compared to the lower altitudes plots. The Jaccard index of dissimilarity decreases when

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the altitude increases. This encounters the statements of Bell (2000) for whom the composition of a community will be highly correlated with that of its immediate successor. Environmental-limitation conditions (lower temperatures, humidity, cloudiness…) created by the altitude act as ecological filtering and inhibit the installation of some species at higher altitudes (Salas-Morales and Meave, 2012). Only some plant species succeed to live in such conditions. This is the reason why the floristic difference between layers diminishes with increasing altitude as many species present in the canopy layer are encountered also in the understorey layers at those altitudes. McEwan et al. (2005) found in the case of their study in an old-growth forest of Kentucky in the USA that layers similarity increases with altitude. Wu et al. (2007) found the same results in the Mount Fuji in Japan.

Variation of the relative abundance of canopy species

After past disturbances in the highlands of the KBNP (Masumbuko, 2011; Kabonyi et al., 2011), the regeneration capabilities of canopy species were less known. The results from this study showed that the most represented species in the canopy are also well represented in the lower layers at higher altitudes (see *Figure 4*). Gonzalez et al. 2010 found that the relative abundance of a given species in two compared layers can highlight some insights on its dynamics and the forest stability.

Successive layers such as sapling/understorey and understorey/canopy are not independent because each one is largely built from survivors and offspring of previous ones (Hall and Swaine, 1976; Bell, 2000). This represents the main interest of studying sapling and or understorey layers which are made of species which may constitute the canopy layer in next generations (Gonzalez et al., 2010). In a dry tropical forest of Costa Rica, Kalacska et al. (2004) found that there are some species which are present only in the early stage of development and are absent in the late stage. Some species can be present in all stages of development but with varying abundances. It has been argued that the appearance or disappearance of species in a given layer (or stage of development) is a great signal in its dynamic trend (McGill et al., 2007), either it is replacing or it is being replaced (McEwan et al., 2005; Zhang et al., 2009; Njunge and Mugo, 2011). Canopy species with high relative abundance in lower layers could maintain their presence in the canopy layer even in next generations (Hall and Swaine, 1976).

Does the light requirement confirm the floristic stability of higher altitudes?

The light requirement of species can be informative of the level of stability of a forest (Pierlot, 1966; Lovett et al., 2006). In the sylvigenetic cycle, early stage of development of a forest is dominated by trees which will be replaced by others, these trees are generally pioneer species (Hallé et al., 1978; Gourlet-Fleury et al., 2005). In the absence of high disturbance, pioneer species characterized by a short life span form a first canopy which constituent will be replaced by non-pioneer light demanding species and/or shade tolerant species which grow beneath this canopy (Whitmore, 1989; Van Gemerden et al., 2003b). When the forest tends to be stable it becomes more dominated by non-pioneer light demanding and shade tolerant species (Hall and Swaine, 1976; Whitmore, 1989; Van Gemerden et al., 2003b; Lovett et al., 2006). This affects the understorey and sapling's composition because of the lack of sunlight causing difficult germination and growth conditions to pioneer species (Harper 1977 in Reed and Foster, 1984). In the case of this study, we find that the number of pioneer species is lower than that of shade tolerant species and non-pioneer light demanding species.

The *Table 2* showed that in all layers the number of pioneer species which are proof of large and recent disturbance (Van Gemerden et al., 2003b) are negatively correlated to the altitude and that non-pioneer light demanding species and shade tolerant species are positively correlated to the altitude. Plots situated at lower altitude are more disturbed than those at higher altitude suggesting that higher altitudes are more stable than lower. As said above, this could be a consequence of past and recurrent political turmoil which affected severely lower altitudes than higher ones.

Conclusion

Like herbaceous species and other organisms, woody plant species richness and diversity decrease with increasing altitude. Both sapling, understorey and canopy species showed this trend. Higher altitudes have low number of species and high number of individuals in the three layers. Plots situated at higher altitudes are more stable than those at lower altitudes. This was confirmed by the result from the Jaccard index of dissimilarity, the comparison of relative abundances and the variation of pioneer and non-pioneer species with the altitude. From the comparison of canopy and understorey or sapling abundances we can predict that the well represented canopy species in the understorey or in the sapling could form the future canopy as long as an equilibrium rate of mortality, regeneration and growth is maintained. Furthermore, we observed that pioneer species which are more abundant in unstable forests are more represented at lower altitudes than they are at higher and that non-pioneer and shade tolerant species increase with the altitude supporting the statements that high altitudes are more floristically stable than lower altitudes. In spite of these conclusions, we suggest that other studies can be conducted to clarify the role of other factors than altitude (geomorphological factors, soil, temperature, cloudiness, humidity, seed dispersal…) in the stability of montane forests.

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EFFECT OF BIOFERTILIZERS ON THE MORPHOLOGICAL CHARACTERISTICS OF THE BURDOCK

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Abstract. Using eleven treatments, the present study aimed at investigating the influence that organic and inorganic fertilizers exert upon the germination percentage, the length and weight (i.e. dry and fresh) of the shoot and root, and number of leaves which are considered the morphological characteristics associated prominently with the burdock that is technically entitled *Arctium lappa*. Significant differences were observed between such treatments as urea, vermicompost, vermicompost combined with Nitroxin, 15% manure in comparison with the control treatment in terms of the germination percentage. Another salient feature of urea and vermicompost treatments was that they led to a decrease in the rate of germination. Vermicompost treatments were proven to be significantly different in terms of number of the plant leaves. Shoot and root length was shown to be significantly influenced by the vermicompost treatments utilized both individually and in combination with Nitroxin. Compared with the control treatment, the experimental treatments were more significant as far as the weight of the fresh root and shoot was concerned. Another source of significant difference in terms of dry root weight was identified to be correlated with the 15% and 30% vermicompost individually used as well as the 15% vermicompost and manure employed in combination with Nitroxin. As for the dry shoot weight, no significant difference was witnessed between Nitroxin and 30% manure treatments.
Keywords: *Arctium lappa, growth parameters, inorganic fertilizer, Nitroxin, vermicompost*

Introduction

Arctium lappa Linne, popularly known as burdock or bardana, is a member of the *Asteraceae* (*Compositae*) family and can be found worldwide (da Silva et al., 2013). This genus is native to Eurasia (Duistermaat, 1997). In Flora Iranica, three studied species are reported in Gilan, Golestan, Mazandaran, Tehran, Khorasan, Kerman, Lorestan and Azerbaijan provinces (Henry, 2002; Karimi et al., 2001). The morphological characteristics of this plant include the straight and branched stems covered with fluff, heart-shaped leaves, and same-genus heaps and the cluster inflorescences or pistil and almost spherical involucres, bracteoles in several bayonet and narrow rows, akene with cut and slightly compressed tip (Ghahreman, 1994). In addition, the root of burdock is long and fleshy, gray-brown outside and whitish inside, with a somewhat thick bark and soft wood tissue with a radiate structure. It has a sweet taste and crisp texture. Burdock (*Arctium lappa* L.) has long been cultivated and commonly consumed as a very popular vegetable in Asia (Imahori et al., 2010). The

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nutrients contained in *A. lappa* include inulin, polyphenols, chlorogenic acid, proteins, carbohydrates, vitamins, amino acids, minerals, and unsaturated fatty acids (Chang et al., 2009). Moreover, in folk medicine, *A. lappa* had been used to treat throat pain, arthritis, rashes, and various skin problems, and also as a diuretic, depurative, and digestive stimulant (Chan et al., 2011). The burdock beneficial effects observed are related to hypertension, gout, arteriosclerosis, hepatitis and other inflammatory disorders (Lieber, 1994; Tamayo, 2000). The several investigators have been demonstrated that *A. lappa* displayed hepatoprotective (Lin et al., 2002); antibacterial properties against gram-positive and gram-negative bacteria (Gentil et al., 2006; Pereira et al., 2005) and anti-inflammatory effects (Zhao et al., 2009), which might be due to its free radicalscavenging activity (Leonard et al., 2006). Many of the biological properties attributed to burdock, including antimutagenicity, anticarcinogenicity, and antiaging, may originate from the antioxidant ability of its component (Mclarty, 1997; Niki et al., 1997; Yang et al., 2001).

Roots from *A. lappa* are popular in the Asian cuisine being widely consumed, whereas the leaves are used as infusions or, externally, as a poultice (Jeelani and Khuroo, 2012). Although the leaves are rich in phenolic compounds (Lou et al., 2010), to which many health benefits are associated (Tamayo et al., 2000; Sun et al., 2011; da Silva et al., 2013), there is a prevalence of investigations on the roots and seeds. Extracts from roots containing several monocaffeoylquinic acids (MCQA) and dicaffeoylquinic acids (DCQA), including several isomers, were reported to have gastroprotective activity (da Silva et al., 2013; Santos et al., 2008). Other compounds were also found in *A. lappa*, including arctiin and arctigenin, caffeicacid, chlorogenicacid, cynarin, rutin, quercitrin, quercetin, luteolin, benzoic and p-coumaricacid (Lou et al., 2010; Liu et al., 2005). They are associated to the medicinal properties of *A. lappa*, such as the lignin arctigenin that exhibited antitumor and antidiabetic activities (Awale et al., 2006), these squilignans isolappaol C, lappaol (C,D,F) and diarctigenin, which have anti-inflammatory activity (Park et al., 2007). Therefore, improving the productivity and quality of *A. lappa* is an ultimate goal.

Biofertilizers are the formulation of living microorganisms which are able to make atmospheric nitrogen available for the plants (Subba Rao, 1993) and which contain microorganisms capable of transforming the nutritive elements from a non-usable form to a usable one through biological processes (Tien et al., 1979). Several bacteria that are associated with the roots of the crop plants can induce beneficial effects on their hosts and often are collectively referred to as PGPR standing for plant growth promoting rhizobacteria (Azarpour et al., 2011). Nitroxin is a biologic nitrogen fertilizer that contains *Azotobacter* and *Azospirillum* (Arun, 2007). *Azotobacter* and *Azospirillum* are the two most important non-symbiotic nitrogen-fixing bacteria in non-leguminous crops. Under appropriate conditions, *Azotobacter* and *Azospirillum* can enhance plant development and promote the yield of several agriculturally important crops in different soils (Okon and Labendera-Gonzalez, 1994). These beneficial effects of *Azotobacter* and *Azospirillum* on plants are attributed mainly to an improvement in root development, an increase in the rate of water and mineral uptake achieved by roots, a displacement of fungi and plant pathogenic bacteria and, to a lesser extent, biological nitrogen fixation (Okon and Itzigshohn, 1995). Furthermore, vermicompost is a product of biodegradation and stabilization of organic materials made by an interaction between earthworms and microorganisms. It contains microbial activities (Edwards, 1998). Vermicompost contains plant-growth regulating materials such as humic acids (Senesi

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et al., 1992; Masciandaro et al., 1997; Atiyeh et al., 2002) and plant growth regulators like auxins, gibberellins, and cytokinins (Krishnamoorthy and Vajrabhiah, 1986; Grappelli et al., 1987; Tomati et al., 1988), which are responsible for the increased plant growth and yield of many crops (Atiyeh et al., 2002).

Therefore, in reference to the mentioned issues, the growth characteristics including the percentage and rate of germination, the number of leaves, roots and shoot height, root and shoot fresh and dry weight of the burdock constituted the focal points of the current study. The effects of Nitroxin (mixed with *Azospirillium* and *Azotobacter*), vermicompost and cow manure bio-fertilizers; chemical fertilizers (i.e. urea) at two levels (15 and 30%) on the morphological characteristics were also examined.

Methods

Context and Materials of the Study

The design of the current study was completely randomized, and it was based on some experiments conducted throughout the study using 11 treatments and 4 replications in a greenhouse located in Isfahan (Khorasgan Branch), Islamic Azad University, Isfahan in Iran in 2013-2014. To achieve the main goal of the study, such fertilizers as vermicompost, Nitroxin (*Azotobacter* and *Azospirillium brasilense*), cow manure and urea as a chemical fertilizer were utilized along with some pots (16*19 cm) containing approximately 2 kg of soil and sand extracted from some farms near the university context mentioned above. The burdock is a biennial (lives 2 years), the most development of leaves and root occurs in second year. However, measurement of vegetative growth of seeds can be determining the deferent fertilizer treatments potential in first year in regard with the potted planting of burdock. In the bottom of pots the holes with a diameter of 1.5 cm is considered, it is to measure the roots that grow larger than the size of the pot. Moreover the pots embedded in an 80 cm height of ground surface (*Fig. 1*).

Figure 1. *Arrangement of pots in the greenhouses*

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Treatments

The pots 1-4, 5-8, 9-12, 13-16, 17-20, 21-24, 25-28, 29-32, 33-36, 37-40, and 41-44 were comprised of some soil and sand as the control sample of the study (i.e. 15% vermicompost and 85% soil, 15% vermicompost and Nitroxin and 85% soil, 30% vermicompost and 70% soil, 30% vermicompost and Nitroxin and 70% soil, Nitroxin and soil, 15% cow manure and 85% soil, 15% cow manure and Nitroxin and 75% soil, 30% cow manure and 70% soil, 30% cow manure and Nitroxin and 70% soil, urea and soil, respectively). After 25 burdock seeds were planted in each pot, and the seeds were covered with one centimeter soil, the pots were once irrigated every two days.

Measurement

The seedlings were counted once a week. On the fourth day, the germinated seeds were counted for 32 days. Finally, after 110 days, the shoot and root length was measured using a transparent ruler, and the normal seedlings were counted again. The leaves of each plant were counted as well. Afterwards, the roots and stems were washed, and the fresh weight was determined. To determine the root and shoot dry weight, the samples were dried in an oven for 48 hours at 72 °C. The dry weight was determined using a digital scale (accuracy level=one ten thousandth of a gram). The germination percentage was calculated using equation (1) below:

$$PG = \frac{Ni}{N} \times 100 \qquad \text{(Eq.1)}$$

Where PG is the germination percentage, Ni indicates the number of the germinated seeds per N day, and N shows the number of seeds planted.

Statistical Analysis

All the experiments were repeated four times. The measured data (Tables of the Appendix 1) were analyzed using the analysis of variance (ANOVA), and the mean values of the treatments were compared using Duncan's Multiple Range Test (DMRT) at P≤0.05. The 19th version of the SPSS was used to analyze the whole data.

Results and Discussion

The results gained from the ANOVA for the vegetative characteristics such as the number of leaves, root and shoot length, fresh and dry weight of root and stem and the germination percentage are shown in *Table 1* below.

Table 1. *Effect of the treatments on the vegetative characteristics of the medicinal burdock plant*

Treatment	% Germination	Number of leaves	Root length (cm)	Stem length (cm)	Root fresh weight (gr)	Shoot fresh weight (gr)	Root dry weight (gr)	Shoot dry weight (gr)
Control	38a	2.75c	12.7a	1.5ef	0.08f	0.09d	0.04f	0.03e
%15 Vermi	39bcd	4ab	28bc	2.87bcd	1.25bc	0.52bc	0.75bc	0.23bcd

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%15 Vermi. Nitroxin	35cd	3.25bc	17.75b	3.6ab	1.4b	0.82c	0.78ba	0.30b
%30 Vermi.	37bcd	4.5a	16.1b	4.25a	2.2a	1.3a	1.1bcd	0.56a
%30 Vermi Nitroxin	16e	3.5abc	15.75bc	3.37abc	0.89bcd	0.78b	0.5f	0.25bc
Nitroxin	24de	3bc	10.1c	0.75f	0.07f	0.07d	0.03def	0.02e
%15FYM	39bcd	2.5c	17.4b	2.1de	0.61def	0.29cd	0.26def	0.12cde
%15 FYM Nitroxin	53ab	3.5abc	17.25b	2.8bcd	0.8cde	0.47bcd	0.43 cde	0.2bcd
%30 FYM	48ab	2.5c	17.75b	2.25cde	0.20ef	0.32cd	0.10 ef	0.11de
%30 FYM Nitroxin	60a	2.75c	16.87b	2.87bcd	0.3def	0.26cd	0.12ef	0.13cde
Urea	0f	-	-	-	-	-	-	-

Means within the column with the same letter are not significantly different by Duncan multiple range test at ≤ 0.05.

Germination Percentage

According to the statistical analysis of the experimental data gained from the studied treatments shown in *Table 1* above, urea made the most significant difference among all the treatments tends by inhibiting the germination process. Urea has many advantages and is widely used as a nitrogen fertilizer throughout the world of agriculture; however, there are problems related to its use (Engelstad and Hauck, 1974). Some of these problems have adverse effects on germination, seed and early plant growth in soil (Gasser, 1964; Goyal and Huffaker, 1984). The adverse effect of urea on germination is due to the presence of impurities such as biuret (Wilkinson and Ohlrogge, 1960), due to cyanate formed by the isomerization of urea in aqueous solution, PH or high concentrations of ammonium ions due to hydrolysis of urea by soil urease (Widdowson et al., 1960; Court et al., 1964), Ammonia production from urea hydrolysis (Court et al., 1964), or nitrite production by nitrification and urea nitrogen by soil microorganisms (Court et al., 1964). Bremner and Krogmeier (1989) reported that the effect of urea fertilizer on seed germination of wheat, rye, barley and maize showed that the adverse effects of urea on seed germination in soil is due to ammonia formed by urease hydrolysis which is not related to urea. Impurities in urea as biuret or nitrite resulted from the nitrification of urea nitrogen. The studies compared the effects of germination on soils made by pure urea, urea and impure urea. Also, the compounds formed as a result of changes in soil microbial and enzymatic urea showed that ammonia volatilization from soils treated with urea completely inhibit the germination process.

Both the vermicompost and the vermicompost combined with Nitroxin showed significant differences in comparison with the control treatment. Also, the two treatments 30% vermicompost combined with Nitroxin and Nitroxin indicated significant differences, but the germination was less than half in the combined treatment. Other treatments including the vermicompost treated both individually and in combination with Nitroxin were not observed to be significantly different from the control treatment. The 15% vermicompost treatment did not reduce the germination level. Once combined, the 15% vermicompost treatment turned out to exert a greater effect on the germination process. It is so likely that in the early stages the plants are susceptible to the negative effects of the vermicompost. The studies showed that the

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application of the vermicompost inhibits the germination and seedling growth so that when the concentration of the vermicompost increases, the growth decreases linearly (Ievinsh, 2011). Buckerfield et al. (1999) reported that radish seed germination decreases gradually once the concentration of the vermicompost increases, but a tenfold increase is observed in the 100% vermicompost as compared with its 10% counterpart.

In the early stages of growth, the plants were susceptible to the negative effects of vermicompost. Moreover, the stability and maturity are essential for successful application (Wang et al., 2004), Stability is related to microbial activity (Lasaridi and Stentiford, 1998). Puberty refers to the degree of decomposition of organic matter during composting and is produced in the absence of pathogens and weed seeds (Wu et al., 2000). Unstable compost leads to the creation of a competition for oxygen uptake between the biomass and the root or seed. The roots and seeds are deprived from oxygen; the H_2S and NO_2 reduce (Mathur et al., 1993). According to Nadi et al. (2011), the germination of pistachio in vermicompost cow manure treatments, compared with the control treatments, showed a significant increase owing to the growth of fungi and the disease in seeds in pots treated by the premature vermicompost. Bachman and Metzger (2008) stated that vermicompost were used to improve the growth and a lack of increase in the percentage or rate of germination caused by new seeds and the effect of vermicompost. Joshi and Vig (2010) reported that the highest germination percentage at 15% vermicompost treatments was observed to be up to 86%, and an increase in the concentrations of the 30 and 45% treatments that were gradually reduced to 60 and 55% owing to the presence of excessive nitrogen that inhibited the germination.

As Saleem et al. (2007) mentioned, Plant Growth Promoting Bacteria (PGPR) including the enzymes of amino cyclopropane and dideaminase carboxylic acid (ACC) facilitates the growth of the plant indirectly by reducing plant pathogens, but directly by facilitating the absorption of nutrients through production phytohormone such as auxins, cytokines and gibberellins and enzymes reducing the level of ethylene. They added that PGPR totally breaks the ACC ethylene precursor to utyrate and reduces ammonia and ethylene levels in growing plants. Throughout their study, Gallardo et al. (1994) demostrated that although a large amount of ethylene was not required for the germination of pea seeds, a certain amount was necessary. A comparison made between the cow manure and control treatments indicated that the 15% cow manure was significantly different from the control treatment, though no difference was found between other sorts of the cow manure treatment and the control treatment. However, the combination of the cow manure treatment and Nitroxin was found to be apt for germination in that the highest germination level was observed to be attributed to the %30 cow manure treatment combined with Nitroxin. Germination increased when the concentrations of the %15 to %30 cow manure treatments increased.

It should be mentioned here that the components of the manure treatment can enhance or hinder the germination process. Studies demonstrate that access to a higher level of nitrogen stimulates the germination of some species (Luna and Moreno, 2009). Using different doses of manure with the purpose of influencing the germination process, Carlos et al. (2013) argued for a threshold level above which cow manure could not be influential in the germination process.

Number of Leaves

In terms of the number of the leaves, no significant difference was found between 30% vermicompost treatment combined with Nitroxin, the 15% vermicompost and the

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15% manure treatments combined with Nitroxin. As indicated by *Table 1*, no significant difference was witnessed between the 15% and 30% cow manure treatments. Through the application of vermicompost for the growth of strawberries, Singh et al. (2008) showed that the vermicompost highly influenced such growth parameters as the leaf width and dry weight. This effect was due to the availability of plant growth regulation and humic acid, which is produced by increasing the activity of microbes in vermicompost (Arancon et al., 2004). Microbes such as fungi, bacteria, yeasts and actinomycetes that produce hormones like auxin and gibberellin make a significant amount of vermicompost (Brown, 1995; Arancon et al., 2004).

Stem Length

The results gained from a statistical analysis of the stem length presented in *Table 1* indicate a significant difference made by the vermicompost and the composition of vermicompost and Nitroxin. The 30% vermicompost treatment which turned out to exert the most significant influence on the stem length exhibited no significant difference from the vermicompost composition, though a significant difference was observed as far as the 15% vermicompost treatment combined with other treatments was concerned. The greatest impact on the stem elongation was found to be related to the 15% and 30% vermicomposts combined with Nitroxin and the 30% vermicompost treatment. The 15% vermicompost was more effective once combined with Nitroxin. Joshi and Vig (2010) demonstrated that the application of 15%, 30% and 45% vermicompost treatments increased the height of tomato more significantly than their control treatments. Gutiérrez-Miceli et al. (2007) contended that a significant increase in the average stem diameter and plant height was caused by the use of different doses of sheep manure vermicompost. High microbial activities in the vermicompost are due to fungi, bacteria and autonomists (Tomati et al., 1988). Microorganisms such as bacteria, fungi, yeasts, algae and actionists cause the production of plant growth regulators such as auxin, gibberellins, cytokinin and ethylene (Frankenberger and Arshad, 1995) and vermicompost showed a positive effect on the growth of *Begonias* and *Coleus* (Tomati et al., 1988).

Nitroxin treatment, as indicated by *Table 1* above, was not significantly different from the control treatments, but it significantly reduced the stem length. The average nitrogen rate of soil or nitrogen by nitrogen fixation is not a barrier to nitrogen fixing but its high levels reduce nitrogen fixing (Marschner, 1995).

The cow manure and its combination with Nitroxin were not observed to be significantly different. This composition had a greater impact on the stem length than the individual application of the cow manure treatment. The differences between the 15 % and 30% cow manure and control treatments were not significant.

Shoot Fresh Weight

As for the fresh weight of the shoots, the vermicompost treatments (i.e. the vermicompost itself and its combined form) were shown to be significantly influential. Specifically, the 30% vermicompost was found to play the most critical role in the weight than other treatments of the study. According to other studies (Peyvast et al., 2008), the greatest impact on the plant growth and yield was demonstrated to be made by low ratios of vermicompost (20 to 40 %). A reduction in shoot fresh weight was observed in the Nitroxin treatment, but there was no significant difference between this

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treatment and the control one. The cow manure treatments were found not to be significantly different in this respect. They had not been different from the control treatment neither. However; the stem was much more significantly affected by this treatment than the control treatment of the study.

Shoot Dry Weight

As for the shoot dry weight, a significant difference between the vermicompost itself and its combined form was found. In addition, the 30% vermicompost was more significantly influential than other treatments. The Nitroxin treatment decreased the shoot dry weight in comparison with the control treatment, yet the difference was not significant. This treatment was significantly different from other treatments, but the difference existing between the 30% cow manure treatments applied individually and the one utilized in combination with Nitroxin was not significant. The greatest impact on stem dry weight was found to be correlated with the combination of the 15% cow manure and Nitroxin.

Root Length

According to *Table 1*, significant differences between the experimental and control treatments were observed. The root length increased due to the 15 and 30% vermicompost treatments. The plants respond differently to different doses of vermicompost due to the production of growth enhancing materials in lower doses than higher doses (Arancon et al., 2004). No significant difference was found between the 15% vermicompost and 15% vermicompost combined with Nitroxin, as well as between the 30% vermicompost treated solely and the one combined with Nitroxin. A root length reduction was observed in 15% and 30% vermicompost treatments combined with Nitroxin. Nitroxin treatments were demonstrated not to be significantly different from the 15% and 30% vermicompost treatments combined with Nitroxin, but the difference was significant between the Nitroxin and other treatments. Also, a significant reduction in the root length occurred due to the experimental treatments in comparison with the control one. Vessey (2003) opined that an increase in gibberellins can increase cell elongation influenced by biological fertilizers. He asserts that rhizosphere bacteria are likely to cause the synthesis and release of auxin as a matter of secondary metabolites in roots.

Cow manure treatments applied individually or in combination with Nitroxin didn’t exert a significant influence on the root length in comparison with the control treatments. Verlinden et al. (2010) conducted a study on the effect of biofertilizers on increasing pasture vegetative organs. The availability of food increased the population and colonization of bacteria in the root of the organic fertilizers combined with *Azotobacter* and *Azospirillium* (Siddiqui, 2004).

Root Fresh Weight

As presented by *Table 1*, the vermicompost treatment applied both individually and in combination with Nitroxin made a significant influence upon the fresh weight of the roots in comparison with the control treatment. The 30% vermicompost was the most significantly influential in the root fresh weight among all the treatments of the current study. This significance can lie in the increasing amount of available nutrients, the number of beneficial microorganisms, enzyme activities and plant growth promoters

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such as gibberellin, cytokinin and auxin. Kadam and Pathade (2014) showed that using the proper proportions of vermicompost, root fresh weight increased more significantly in the experimental treatment than its control counterpart. In their study, the Nitroxin treatment showed no significant differences, but it led to a higher reduction in the root fresh weight in comparison with the control treatment. The cow manure treatments were greatly but insignificantly effective in the fresh weight as compared with the control treatments.

Root Dry Weight

Regarding the root dry weight, the vermicompost treatments, especially the 30% form, had a more significant influence than the control treatment. Bachman and Metzger (2008) examined the effect of 10% and 20% vermicompost treatments on marigold, revealing that the latter brought about a greater increase in shoot and root dry weight than the former. In their study, the Nitroxin treatment showed no significant differences as compared to the control treatment. The cow manure treatments, especially the 15% form, and their combination with Nitroxin were more significant than other treatments in this respect.

Conclusions

The results gained from the current study showed different effects of application of vermicompost, biological, cow manure and urea fertilizers on the germination process. The germination percentage of the vermicompost and Nitroxin decreased in comparison with the control treatment. The cow manure treatment and its combination with Nitroxin increased the germination percentage. Furthermore, a higher dose of the vermicompost treatment was found to be more influential in the number of the leaves. Both levels of the cow manure treatment turned out to be negatively influencial in the number of the leaves. A negative effect was found to be made by the Nitroxin treatment on the shoot fresh weight. Regarding the shoot dry weight, all the study treatments had a great effect except for Nitroxin. In terms of the root length, all treatments were significantly different in comparison with the control treatment. The 15% vermicompost applied individually, the 15% vermicompost used in combination with Nitroxin, and the 30% cow manure were witnessed to be the most significantly influential, but the Nitroxin was found to have a negative influence. Of all the treatments included in the present research study, the 30% vermicompost treatment had the highest effect on the root fresh weight which was differently influenced by the experimental treatments (i.e. vermicomposts) and the control treatment. The root dry weight was shown to be positively significantly influenced by the 15% and 30% vermicomposts, and the 15% cow manure treatment combined with Nitroxin. More importantly, the vermicompost treatments applied individually and in combination with Nitroxin had the greatest effect among all the treatments. The 30% vermicompost was observed to be the highest in this respect. The organic manures and their combination with biofertilizers played a crucial role in the growth of burdock. As for the number of the leaves, stem length, fresh and dry weights of the roots and shoots, the higher doses of the vermicompost 30% were more influential than the 15% vermicompost treatment. Regarding the characteristics such as the germination percentage, the number of the leaves, and the length of the roots, the 15% vermicompost assumed the most significant role, whereas the combined version of the vermicompost treatment was most influential in other parameters.

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The individual and non-individual application of the 15% vermicompost treatment had a negative effect, so this level was proved to be far from optimal as far as the morphological characteristics were concerned. The negative impact of Nitroxin treatment (containing *Azotobacter* and *Azospirillium*) was probably due to the negative interplay between two bacteria. The 15% cow manure applied both individually and non-individually was significantly influential in all parameters except for the root length, but the 30% counterpart had the highest impact on all the features of the plant. Consequently, it is suggested that a lower dose of the animal manure be applied in order to enhance the growth of burdock. However, as for the germination percentage, and the shoot and root dry weight, the combined form is highly recommended. A lower dose of other treatments combined with Nitroxin is suggested once other parameters are intended to be taken into consideration.

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APPENDIX

Appendix 1. *Tables of burdock biometric variation in different treatment*

Pot	Root height (cm)	Stem height (cm)	Root fresh weight (gr)	Stem fresh weight (gr)	Dry root weight (gr)	Dry stem weight (gr)	Number of leave	Germination%
1	8	1.5	0.07	0.06	0.03	0.04	4	44
2	17	1.5	0.11	0.12	0.05	0.05	3	40
3	15.5	2	0.11	0.11	0.08	0.05	2	40
4	10.5	1	0.06	0.07	0.01	0.01	2	28
5	34	3.5	1.2	0.53	0.71	0.2	4	32
6	22	3.5	1.12	0.65	0.73	0.24	4	36
7	29	2.5	1.6	0.45	0.84	0.23	4	40
8	27	2	1.11	0.48	0.73	0.27	4	48
9	17	3	1.12	0.56	0.6	0.22	3	32
10	18.5	3.5	1.45	0.76	0.74	0.25	3	36
11	17.5	4	1.85	0.82	1.1	0.31	3	32
12	18	4	1.23	1.15	0.69	0.44	4	40
13	18.5	4.5	2.12	0.95	1.15	0.42	5	52
14	15	4	2.85	1.12	1.54	0.54	5	36
15	16.5	4.5	1.46	1.4	0.71	0.49	4	32
16	14.5	4	2.46	1.9	1.39	0.79	4	28
17	15.5	3.5	2.21	1.64	1.25	0.46	3	20
18	17.5	3.5	0.31	78.2	0.17	0.22	3	16
19	18	5	0.83	0.51	0.41	0.25	5	16
20	12	1.5	0.21	0.22	0.17	0.09	3	12
21	9.5	0.5	0.09	0.11	0.05	0.02	3	40
22	7.5	0.5	0.02	0.03	0.01	0.01	2	4
23	9.5	1	0.08	0.09	0.03	0.05	4	28
24	14	1	0.09	0.06	0.04	0.03	3	24
25	13	1.5	0.25	0.16	0.15	0.05	3	60
26	24	2.5	0.68	0.31	0.38	0.11	2	52
27	18.5	2	0.78	0.26	0.21	0.14	2	20
28	14	2.5	0.74	0.46	0.32	0.18	3	24
29	13.5	2.5	0.74	0.35	0.43	0.16	4	40
30	13.5	3	0.72	0.36	0.39	0.15	3	60
31	17	3	0.81	0.62	0.41	0.25	3	60
32	25	3	0.95	0.57	0.49	0.26	4	52
33	22	3	0.36	0.54	0.21	0.19	3	48
34	20.5	2.5	0.15	0.39	0.09	0.11	3	56
35	17.5	3	0.31	0.35	0.11	0.14	3	48
36	11	0.5	0.01	0.02	0.001	0.001	1	40
37	15.5	2	0.47	0.34	0.25	0.23	2	64
38	20	2.5	0.39	0.27	0.16	0.11	3	60
39	17	3	0.31	0.21	0.06	0.05	3	56
40	15	4	0.03	0.23	0.01	0.12	3	60

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Appendix 2. *The effect of treatment on burdock growth parameters*

Pot	Root height (cm)	Stem height (cm)	Root fresh weight (gr)	Stem fresh weight (gr)	Dry root weight (gr)	Dry stem weight (gr)	Number of leaves	Germination%
1	34	3.5	1.2	0.53	0.71	0.2	4	32
2	22	3.5	1.12	0.65	0.73	0.24	4	36
3	29	2.5	1.6	0.45	0.84	0.23	4	40
4	27	2	1.11	0.48	0.73	0.27	4	48
5	28	2.875	1.2575	0.5275	0.7525	0.235	4	39
6	9.5	0.5	0.09	0.11	0.05	0.02	3	40
7	7.5	0.5	0.02	0.03	0.01	0.01	2	4
8	9.5	1	0.08	0.09	0.03	0.05	4	28
9	14	1	0.09	0.06	0.04	0.03	3	24
10	10.125	0.75	0.07	0.0725	0.0325	0.0275	3	24
-	-	-	-	-	-	-	-	-
11	13	1.5	0.25	0.16	0.15	0.05	3	60
12	24	2.5	0.68	0.31	0.38	0.11	2	52
13	18.5	2	0.78	0.26	0.21	0.14	2	20
14	14	2.5	0.74	0.46	0.32	0.18	3	24
15	17.375	2.125	0.6125	0.2975	0.265	0.12	2.5	39
	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-
16	13.5	2.5	0.74	0.35	0.43	0.16	4	40
17	13.5	3	0.72	0.36	0.39	0.15	3	60
18	17	3	0.81	0.62	0.41	0.25	3	60
19	25	3	0.95	0.57	0.49	0.26	4	52
20	17.25	2.875	0.805	0.475	0.43	0.205	3.5	53
-								
21	22	3	0.36	0.54	0.21	0.19	3	48
22	20.5	2.5	0.15	0.39	0.09	0.11	3	56
23	17.5	3	0.31	0.35	0.11	0.14	3	48
24	11	0.5	0.01	0.02	0.001	0.001	1	40
25	17.75	2.25	0.2075	0.325	0.10275	0.11025	2.5	48
26	15.5	2	0.47	0.34	0.25	0.23	2	64
27	20	2.5	0.39	0.27	0.16	0.11	3	60
28	17	3	0.31	0.21	0.06	0.05	3	56
29	15	4	0.03	0.23	0.01	0.12	3	60
30	16.875	2.875	0.3	0.2625	0.12	0.1275	2.75	60

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Appendix 3. *Cumulative data of pot replication in different treatment*

Pot number	8	12	16	20	24	28	32	36	40	sum	gmt
1	0	2	3	2	1	1	1	0	0	10	
1	0	24	48	40	24	28	32	0	0	196	19.6
2	0	3	2	2	1	1	1	1	0	11	
2	0	36	32	40	24	28	32	36	0	228	20.72727
3	1	1	0	1	2	2	2	1	0	10	
3	8	12	0	20	48	56	64	36	0	244	24.4
4	0	2	2	0	1	1	1	0	0	7	
4	0	24	32	0	24	28	32	0	0	140	20
5	1	1	1	2	1	2	1	0	0	9	
5	8	12	16	40	24	56	32	0	0	188	20.88889
6	0	1	1	1	0	1	1	2	2	9	
6	0	12	16	20	0	28	32	72	80	260	28.88889
7	0	1	3	1	2	1	2	1	0	11	
7	0	12	48	20	48	28	64	36	0	256	23.27273
8	0	1	1	1	2	3	1	1	1	11	
8	0	12	16	20	48	84	32	36	40	288	26.18182
9	0	1	1	2	0	1	1	1	1	8	
9	0	12	16	40	0	28	32	36	40	204	25.5
10	0	1	1	0	1	1	2	3	0	9	
10	0	12	16	0	24	28	64	108	0	252	28
11	0	1	1	2	1	1	2	0	0	8	
11	0	12	16	40	24	28	64	0	0	184	23
12	0	1	1	0	2	2	3	1	1	11	
12	0	12	16	0	48	56	96	36	40	304	27.63636
13	0	1	1	1	1	2	3	4	0	13	
13	0	12	16	20	24	56	96	144	0	368	28.30769
14	0	1	1	1	1	2	3	1	0	10	
14	0	12	16	20	24	56	96	36	0	260	26
15	0	0	1	1	1	2	2	1	0	8	
15	0	0	16	20	24	56	64	36	0	216	27
16	0	1	1	2	2	1	1	0	0	8	
16	0	12	16	40	48	28	32	0	0	176	22
17	0	0	1	1	1	1	2	0	0	6	
17	0	0	16	20	24	28	64	0	0	152	25.33333
18	0	2	1	0	0	1	1	0	0	5	
18	0	24	16	0	0	28	32	0	0	100	20
19	0	1	1	0	0	1	2	0	0	5	
19	0	12	16	0	0	28	64	0	0	120	24
20	0	1	0	0	0	0	1	1	0	3	
20	0	12	0	0	0	0	32	36	0	80	26.66667
21	0	1	2	1	1	3	2	1	1	12	
21	0	12	32	20	24	84	64	36	40	312	26
22	0	1	1	0	0	0	0	0	0	2	
22	0	12	16	0	0	0	0	0	0	28	14
23	0	2	1	1	0	2	1	1	0	8	
23	0	24	16	20	0	56	32	36	0	184	23
24	0	1	1	1	1	2	1	0	0	7	
24	0	12	16	20	24	56	32	0	0	160	22.85714
25	0	2	4	3	0	3	2	2	1	17	
25	0	24	64	60	0	84	64	72	40	408	24
26	1	1	1	3	3	4	1	0	0	14	
26	8	12	16	60	72	112	32	0	0	312	22.28571

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27	0	1	1	1	0	2	0	0	0	5	
27	0	12	16	20	0	56	0	0	0	104	20.8
28	0	1	1	1	2	1	1	0	0	7	
28	0	12	16	20	48	28	32	0	0	156	22.28571
29	1	1	1	2	3	1	1	1	0	11	
29	8	12	16	40	72	28	32	36	0	244	22.18182
30	1	2	1	2	1	3	2	1	2	15	
30	8	24	16	40	24	84	64	36	80	376	25.06667
31	1	1	4	3	2	1	2	1	1	16	
31	8	12	64	60	48	28	64	36	40	360	22.5
32	3	3	2	2	1	0	1	1	0	13	
32	24	36	32	40	24	0	32	36	0	224	17.23077
33	3	2	1	1	1	0	0	0	0	8	
33	24	24	16	20	24	0	0	0	0	108	13.5
34	3	2	2	1	2	2	2	1	0	15	
34	24	24	32	20	48	56	64	36	0	304	20.26667
35	1	1	1	2	2	2	3	1	0	13	
35	8	12	16	40	48	56	96	36	0	312	24
36	1	2	1	1	2	1	2	1	0	11	
36	8	24	16	20	48	28	64	36	0	244	22.18182
37	2	3	2	2	2	1	2	2	1	17	
37	16	36	32	40	48	28	64	72	40	376	22.11765
38	0	2	3	2	1	3	2	1	1	15	
38	0	24	48	40	24	84	64	36	40	360	24
39	2	2	2	2	1	1	2	1	0	13	
39	16	24	32	40	24	28	64	36	0	264	20.30769
40	2	3	2	3	2	2	1	1	0	16	
40	16	36	32	60	48	56	32	36	0	316	19.75
41	0	0	0	0	0	0	0	0	0		
42	0	0	0	0	0	0	0	0	0		
43	0	0	0	0	0	0	0	0	0		
44	0	0	0	0	0	0	0	0	0		

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ESTIMATING THE POTENTIAL SUSTAINABILITY OF GEOSYSTEMS IN CONDITIONS OF ANTHROPOGENIC IMPACTS (A CASE STUDY OF SARYSU BASIN, KAZAKHSTAN)

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Abstract. Results of estimating geosystems'sustainability in conditions of anthropogenic impacts are presented in this work. The method for assessing the potential sustainability of geosystems in conditions of anthropogenic impact was improved and adapted. Integral assessment of the sustainability of geosystems of Sarysu Basin, Kazakhstan with regard to human impact by identifying landscape factors, allowed the differentiation of geosystems into 5 classes, requiring different approaches to the development of optimal environmental management structures.
Keywords: *basin, landscape, sustainability, anthropogenic impact, geographic information systems*

Introduction

The question of geosystem sustainability is becoming increasingly important in the case of increasing anthropogenic influence on the environment. Geosystems need to be researched, and to predict their conditions as a result of external factors to increase further production growth. The selection and implementation of the proper forecast methodology is only possible in static equilibrium systems. The development of the system in geosystems which are in a condition of crisis and in which an unstable equilibrium is emerging, can take an unpredictable path and lead to catastrophic consequences.

The concept of sustainability in physical geography has no clear definition. The definition depends on the purpose of assessment of natural or human-modified geosystems, which is given by different authors. All interpretations of this concept can be summarized in the following components: limits of natural functionality, ability to resist external influences (natural and anthropogenic), and ability to relax after load reduction (Chorley et al., 1971; Ryumin, 1990; Bastian et al., 2002). According to Sochava (1971), all the dynamic changes which take place within a single invariant (qualitatively unchanged) are an example of geosystem sustainability, because this represents the ability to restore to a previous condition or position. According to Ryumin (1990), a proper functioning of the system is linked with the phenomena of seasonal dynamics and has second importance in the definition of the sustainability. On the other hand, Isachenko (1980) emphasizes the role of dynamics in geosystems' sustainability.

The research into geosystem sustainability in the face of anthropogenic pollution and the self-purification ability of technogenic materials plays an important role in landscape geochemistry (Solntseva, 1982; Glazovskaya, 1983; Volkova et al., 1987; Turner et al., 2001; Fu et al.,2013; Wu, 2013; Michaeli_et al., 2014). The stability of geosystems with regard to technogenesis is described by Glazovskaya (1997), via the self-purification ability. This ability is conditioned by the speed of transformation of man-made substances and their removal beyond geosystems (Glazovskaya, 1983). In many ways, this ability is possible owing to the compatibility of natural and man-made substance flows (Solntseva, 1982; Shadrina et al., 2014; Yermolaev et al., 2014; Michaeliet al., 2015).

Research into the sustainability of geosystems of the Sarysu Basin focuses on the increasing human impact on its environment. In this regard, the aim of the study of selected areas is determined by the necessity to obtain new landscape environmental information demanded for the future creation of the Sarysu Basin nature management strategy.

Methods

There is potential and real sustainability of the landscape. The first concept refers to the natural (undisturbed) condition; the second to a modern condition, absorbed by all layers that have accumulated in the history of human impact (Benson et al., 2007). However, it should be noted that the starting point for assessing the current sustainability of geosystems for forecasting development and work should be the initial variant, as the object of the basic landscape classification on the basis of its sustainability.

We analyse the geosystem's sustainability as the ability to maintain its structure and function under external impact. We used the principles of assessing the sustainability of the soil and the landscape, created by Glazovskaya (1997), Bashkin and Evstafieva (1993), and Orlova (2002; 2006) (*Table 1*), to assess the sustainability of geosystems with regard to human impacts. These principles of assessment are based on normalizing methods of individual indicators, and their subsequent summation on a point system. They take into account the complex for an integrated assessment of sustainability of the whole group and geosystems, in terms of their overall sustainability (*Table 1*). The authors are fully aware that the proposed method of assessment of geosystem sustainability is only one of several possible practical approaches, which realization demands a lot to go through.

Table 1. *The scale of mark estimation potential of landscape sustainability in conditions of anthropogenic impacts (compiled from materials of Glazovskaya (1997) Bashkin, Evstafieva (1993), Orlova 2002; 2006)*

Indicators	**Scores Of Sustainability**				
	1 score	**2 score**	**3 score**	**4 score**	**5 score**
Radiation balance, kcal/cm^2 per year	5–10	11–20	21-30	31–50	more than 50
Radiation index of dryness	less than 0.45 and more than 3	-	1.01. or 3.00	-	0.45–1.00
Wind conditions (amounts of days with strong wind)	more than 51	-	21–50	-	0.45–1.00

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Wind conditions (amounts of days with strong wind)	more than 51	-	21–50	-	less than 20
Nature of the landform	hilly	hilly steeply sloping terrain	gently hilly terrain	plateau and slightly wavy terrain	plain
Downhill gradient, degree	more than 20	5.1–20	3.1–5	1.1–3	0–1
Geochemical location	accumulative	-	Transit	-	eluvial
The rate of natural drainage	>0.0005very poorly drained	a little bit poorly drained 0,0005-0,001	poorly drained 0.001–0.008	drained	intensive drained
Level of hydromorphic soil	hydromorphic	-	semi-hydromorphic	-	automorphic
Texture of soil	sand	clay sand	light loam	medium loam	heavy loam
Humus horizon power, cm	less than 3	3–10	10.1–25	25.1–80	more than 80
Humus content in horizon 0–20 cm, %	less 2	2.0–4.0	4.1–6.0	6.1–9.0	more than9.0
Acidity status of the soil (pH)	very acid soil (4.5 and less) or very alkaline soil (8.5 and more)	acid (4.5–5.0) or alkaline (7.5–8.5)	slightly acid soil (5.0–5.5) or slightly alkaline soil (7.0–7.5)	near to neutral (5.5–6.0)	neutral (6.0–7.0)
Salinity (the amount of salt in the upper layer of horizons, %)	highly saline and very saline (0.6)	moderately saline (0.3–0.6)	slightly saline(0.2–0.3)	very slightly saline(0.15–0.2)	non-saline (less than 0.15)
Cation exchange capacity, mg.ekv /100 gr. soil	less than 10	10–20	21–30	31–40	more than 40
Type of water regime	desuctive-exudational regime	exudational regime	non-leaching regime	periodically leaching regime	leaching regime
Square canopy %	Lesst han 20	20–40	41–60	61–90	more than 90

We have to pay attention among climatic factors to some important factors determining energy flow in geosystems, such as radiation balance, irrigation adequacy, and wind conditions.

Radiation balance is determined by the relationship between the amount of energy reaching an object (or a portion of it) and the amount leaving it. The value of the radiation balance depends on many factors: latitude, surface properties and surface moistening, which have a direct impact on albedo and effective terrestrial radiation. The energy of the main biogenic and abiogenic processes in geosystemsis determined by radiation balance. According to Glazovskaya (1983), the speed and direction of chemical transformations of technological products is determined by radiation balance as well. Moreover, large parameters of radiation balance coincide with the maximum geosystem sustainability (under *ceteris paribus*) (Bashkin et al., 1993).

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The radiation index of the dryness value of dryness index characterizes the lack of moisture and is considered the lowest rate of sustainability (less than excessive moisture). From moisturizing performance, very informative radiation dryness index (K), the proposed M.I. Budyko (1984), which is the ratio between the radiation balance of the territory and the annual amount of precipitation, expressed in calories latent heat of vaporization, the equation (*Eq. 1*):

$$K = R/LQ \qquad \text{(Eq. 1)}$$

R - radioactive balance per year, kilocalorie/cm^{2}per year;
L - latent energy of vaporization, kilocalorie/cm^{2}per year;
Q - annual amount of precipitation, mm.

With K=1 the possibility of vaporization is approximately around the amount of fallen humidity. This is relevant to conditions of maximum geosystem sustainability. With R/LQ less than 0.45 to 1 climate is called a damp climate, with K from 1 to 3 it is temperate climate , with K more than 3 it is an arid climate. Radiation index of dryness reflects water and humidity conservation with radiation conditions (Reymers, 1990).

The wind regime, on the one hand, is the factor of dispersion of artificially produced elements in the air, but on the other hand, it is also the factor of lateral (eolian) processes, and determines the adaptation features of biota. The number of days with strong wind per year are used as quality indicators to estimate the sustainability of geosystems.

The features of relief are used as an indicator which represents age and stage of development of the geosystem and compliance with endogenous and exogenous processes. Flat, uniform, slightly wavy and sleepy, sloping terrains have the highest degree of sustainability, whereas hilly terrains have the lowest one.

A steep slope plays an important role concerning geosystem sustainability, because an increase in steepness leads to more intense surface run-off, which increases the risk of the mechanical demolition of solid substances. Therefore this leads to the development of soil erosion. In addition, the speed of the real steep slope influences the speed of the real growth of the humus soil horizon, and the speed of the geosystem's recovery on slopes depends on thesteep slope. The angle of repose, the angle between the ground surface after land subsidence and floor sliding and the horizon are used to characterize the steep slope (Glazovskaya, 1997).

The important factor to determine the sustainability of geosystems from human impact is the determination of its geochemical position, which characterizes the nature and intensity of migration flows. Glazovskaya (1983, 1997) highlights three main types of gradation and two transition types, based on the classification of landscape types. Eluvial (watershed) landscapes are the most highly located, and are geochemically autonomous. They obtain their flow of material from the atmosphere. Transit landscapes are located on the lower stage of the cascade and geochemically are subordinates of elementary landscapes. This landscape obtains its flow of substitutes from the atmosphere and water release from surface and ground waters located higher than some stages of cascade. Accumulative landscapes are usually located on the flat areas near slopes, close to water bodies and floodplains, where there is an accumulation of substances. The accumulative landscapes located in the zone of storage of all incoming substances are the least stable.

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The level of natural drainage of geosystems leads to the process of accumulation or leaching out of chemicals. We used a method of evaluation of morphometric parameters of the basin created by Uglanov (1981), to calculate the natural drainage of geosystems (*Eq. 2*):

$$\mathrm{P} = i\frac{H}{F}, i = \frac{h_1 - h_2}{l} \qquad \text{(Eq. 2)}$$

P - natural drainage;
H - the total length of all the elementary streams that are the basis of erosion (including their length) km;
F - area of the basin, km^2; *i*- main angle of site;
h_1–h_2- height difference of elementary watercourse from its source to its mouth (depth oferosional dissection);
l - watercourse length, km.

The natural drainage classification, including the existing one but with some changes, was created by the authors during the analysis of the literature dedicated to drainage. Thus, geosystems are evaluated according the geosystems' sustainability to drainage: P 0.01–0.2 is very poorly drained; P 0.2–1 is poorly drained; P 1–3 is moderately drained; P 3–10 is well-drained; P 10 and higher is intensively drained.

Soil has a junction place, and forms a natural part of the ground cover's natural components. Indetermining the sustainability of the soil, we proceeded from the fact that it is formed by a buffer capacity (the ability to 'take on' neutralizing this effect, and due to external factors' ability to 'throw off' the load on to other ecosystems, due to the situation in catena, due to climate features) (Vasilevskaya et al., 1997). Next we used soil-geochemical indicators to assessthe sustainability of natural systems to human impact: the rate of hydromorphic soils, texture of soil, depth of humus horizons, the rate of soil acidity, and cation exchange capacity.

Next were groups of soil, depending on the location in the relief and the nature of hydration, called the rows of humidification: automorphic soil formed on flat surfaces and slopes in conditions of free surface water flow, with a deep water table (below 6 m); semi-hydromorphic soil formed by the brief stagnation of surface water or groundwater occurring at a depth of 3–6 m (capillary fringe can reach the roots of plants); hydromorphic soils formed under conditions of prolonged stagnation of surface water or groundwater occurring at a depth of less than 3 m (capillary fringe may reach the soil surface). Rows of humidification of geosystems characterize the geochemical sustainability, which largely determines the intensity of the migration of chemical substances.

The texture of the soil is important for the soil's porosity, air and water permeability, hygroscopicity, absorption capacity, soil temperature, and others (Dobrovol'skiy, 1989). Loam and heavy loam have the best aforementioned indicators than sand and sandy loam.

The rate of soil sustainability to a variety of physical and mechanical stresses, and erosive and deflationary processes is determined by the depth of the humus horizons of the soil (Snakin et al., 1993). The content of humus in the soil largely determines the absorptive capacity of the soil and affects the formation of the structure of the upper soil layers and their physical features. Soils with high humus content can largely resist external impacts.

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The level of soil acidity (the reaction of medium, pH) characterizes many genetic and industrial types of soil. Soils can react differently to pollution products depending on their acidic properties. The mobility of chemical elements and their chemical compounds can be changed in different environments.

The cation exchange capacity (CEC) (the amount of absorbed bases and hydrogen ions) is a crucial characteristic of the soil. It consists of the absorptive capacity of human substances, mineral soil particles, as well as the microorganisms that it includes. The value of soil CEC is correlated with the content of humus in it, the granulometric and mineralogical composition and the level of pH. Soil has a buffering capacity depending on the amount of soil ionic exchange. Hence it has different resistance to external effects (Bashkin et al., 1993).

The type of water regime characterizes the geochemical sustainability of the soil, which is largely determined by the intensity of the removal of substances outside this landscape, the rate of scattering these to the surface, as well as underground drainage and air flow. Based on the classification of types of water regime, Vysotsky and Rode (1965) is necessary to allocate: leaching type and periodical leaching regime (as an intermediate form), non-leaching, and exudative and desuctive-exudational (or stagnation). Leaching type leads to the removal of pollution products; exudative, desuctive-exudational regimes lead to an accumulation of pollution products in the soil profile.

The canopy also helps to reduce soil degradation from erosion. Geosystems covered with a large area of canopy are more resistant to external impact than areas without canopy.

Integral assessment of the sustainability of geosystems to human impacts was obtained by summing the ratings analysed parameters within the operating units of study, which in our work is the landscape. It is based on previously made contact medium-scale landscape map (1:5000 of the Sarysu Basin (*Figure 1*; *Table 2*), which allocated 58 individual landscapes as a result of its typological groups, and also based on the result of the following structural-genetic classification, where groups are arranged in a hierarchical taxonomy: class (plain and mountain landscapes), types (semi-desert and desert landscapes), subtypes (north-desert and south-desert landscapes).

The maximum possible score that characterizes the highest relative sustainability for the area was set at 100%; all other points are expressed as a percentage, which was carried out by recalculating the total points according to the equation presented in Orlova (2002). See equation (*Eq. 3*):

$$C = \frac{100 \sum_{g=1}^{n} Cg}{Q} \quad \text{(Eq. 3)}$$

C - Assessment of potential sustainability of geosystems to anthropogenic influences, %;
Cg - score for each indicator;
Q - maximum possible amount of points;
g - serial number indicator;
n - number of indicators.

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As a result, the following were identified as a Geosystems Group, where grading was based on the total points (%): relatively sustainability geosystems, 100–90; average sustainability, 90– 80; weak sustainability, 80–70; low sustainability. less than 70.

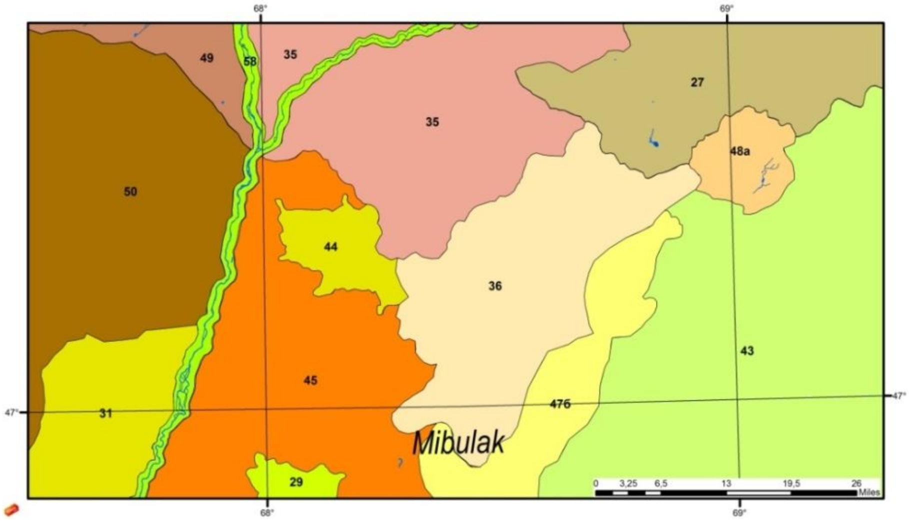

Figure 1. *Landscape map of Sarysu Basin, Kazakhstan (fragment)*

Results

According to the integral estimation of geosystem sustainability we could define that geosystems with the relatively degree of sustainability (landscapes 12, 14, 25, 26, 27, 33, 35, 57) spread over 19.69% of the basin. Geochemical landscapes have an eluvial position with two rather different major landforms: hilly terrain with different slopes (especially 2–6°); and geosystems characterized by relatively favourable thermal and hydrothermal conditions. Dominated by medium and heavy loam automorphic soils, characterized by a non-leaching water regime, non-saline and with neutral or close to neutral the reaction of the soil solution. The humus content inthe layer of 0–20 cm is on average 2.3%, humus horizon is around 12–38 cm. The Cation Exchange capacity is around 16–17 mg.ekv /100 gr. soil. The canopy area is around 70–75% (*Table 3*; *Figure 2*).

Table 2. *Keywords for fragment of Sarysu Basin's landscape map (№ by map Figure 1)*

№	№ **The name of landscapes**
I	**Plain landscapes** **Semi-desertic** **2** ***Aggraded plains*** 27 – Diluvial-proluvial slightly inclined plain with riverbed temporary streams formed by volcanic sedimentary rocks with artemesia-stipa flora on light-brown normal soils.
II	**Desert**
a	**North desert**

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1	***Denudation plains*** 29 – Rolling-wavy socle plain with closed basins and erosional outliers formed by clays, sandstones, sands with grass-Salsolaarbusculiformi, anabasis salsa and tas-anabasis-salsa flora on brown desert soils. 31 – Gently inclined slightly undulating plain with small sand ridges formed by clays, loams, standstones with Salsolaarbusculiformi, anabasis salsa and tas-anabasis-salsa flora on brown desert soils. 35 – Dissected plain built on horizontal strata with closed basins and erosional outliers formed by limestones, dolomites, sandstones with artemesiatas-anabasis-salsa flora on brown desert soils.
2	***Aggraded plains*** 36 – Lake: alluvial ruffed plain formed by limestones, siltstones, porphyries, tuffs with anabasis sals and artemesia flora on brown solonetzic combined with takyrs soils. 43 – Diluvial-proluvial gently-wave plain formed by clays, loams, gravel-pebbles with grass-Salsolaarbusculiformi, anabasis salsa and tas-anabasis-salsa flora on brown desert soils. 44 – Diluvial-proluvial slightly inclined plain formed by clays, sandstones, sands with shrub thickets flora on brown desert soils. 45 – Diluvial-proluvial gently inclined plain with riverbed temporary streams formed by clays, sandstones, sands with wheatgrass, artemisia terrae-albae-ceratoides flora on brown desert soils. 47 – Eolian hilly-ridge plain with small elevations, with artemisia terrae-albae-ceratoides and fescue flora on sands with floodplain meadow soils.
б	48 – Eolian hilly plain with small elevations with white wormwood-fescue and wheatgrass and grey artemisia flora on the sands with floodplain meadow soils
1	**Southern Desert** ***Denudation plains*** 49 – Rolling-wavy socle plain with closed basins and erosional outliers with anabasis salsa and artemesia flora on solonetz. 50 – Undulating plain with closed basins and erosional outliers with grass-Salsolaarbusculiformi, anabasis salsa and tas-anabasis-salsa flora on brown desert soils with solonetz.

Geosystems which have an average degree of sustainability occupied 35.68% of the basin (landscapes 1, 2, 3, 4, 7, 10, 13, 15, 16, 18, 19, 21, 29, 31, 45, 49, 55, 55b, v). Mostly they are hilly and uplands-ridged-sloping plains with low mountain terrain. The geochemical geosystems have an eluvial position with different surface slopes (mostly 0–5°) and low mountain (4 –21°) dominated by medium and light loam automorphic soils, characterized by a leaching water regime, slightly saline, and with neutral or slightly acidic and slightly alkaline reaction of the soil solution. The humus content in the layer of 0–20 cm is around 2.1%, humus horizon is around 15–20 cm. The cation exchange capacity is around 15–16 mg.-ekv / 100gr soil. Hollow sleepy sloping plain is almost completely ploughed up, and uplands-ridged low mountains have an average degree of canopy.

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Table 3. *Sustainability indicators of geosystems of Sarysu Basin in conditions of human impacts (1–4 of 58 indicator fragments)*

Numberof landscapes by map(figure 1)	**Radiation balance, kcal/cm² per year**	**Radiation index ofdryness**	**Wind conditions (amounts of days with strong wind)**	**Nature of the landform**	**Down-hill gradient, degree**	**Geochemical location**	**The rate of natural drainage**	**Level of hydromorphic soil**	**Texture of soil**	**Humus horizon power, cm**	**Humus contentin horizon 0-20 cm, %**	**Acidity status of the soil (pH)**	**Salinity (the amount of salt in the upperlayer of horizons,%)**	**CationExchange capacity, mg.ekv /100 gr. soil**	**Type of water regime**	**Square of canopy %**
1	29.01	5.2	13	ruefully–hilly	3.5	eluvial	0.008	automorphic	lightloam	9–10	2.3	8.2	0.09	17.5	non-leaching regime	30
2	28.68	4.7	26	ridge–arched	3.9	eluvial	0.0007	automorphic	lightloam	9-10	2.4	7.8	0.04	13	non-leaching regime	65
3	29.06	4.9	13	ruffed–hilly	2.9	eluvial	0.008	automorphic	lightloam	9–10	2.1	7.6	0.04	13	non-leaching regime	*70*
4	29.01	5.2	13	ruffed	3.5	eluvial	0.008	automorphic	lightloam	9–10	2.3	8.2	0.09	17.5	non-leaching regime	30

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Geosystems which have a weak degree of sustainability occupied 41.26% of the basin (landscapes 5, 8, 9, 11, 17, 24, 28, 30, 32, 34, 36, 42, 43, 50, 51). The geochemical geosystems has a transit and accumulative position mainly with flat terrain and slopes of surface of around 2°, dominated by hydromorphic loamy and sandy loam soils, characterized by an exudative water regime, moderately saline, and with an alkaline reaction of the soil solution. Depending on the type of soil humus content can change in the layer of 0–20 cm in the scope of values reaches 10.5 and is 1.5–12%, humus horizon is around 10–11 cm. The cation exchange capacity is around 15 mg.-ekv./100 gr. soil. The canopy area is around 55–65%.

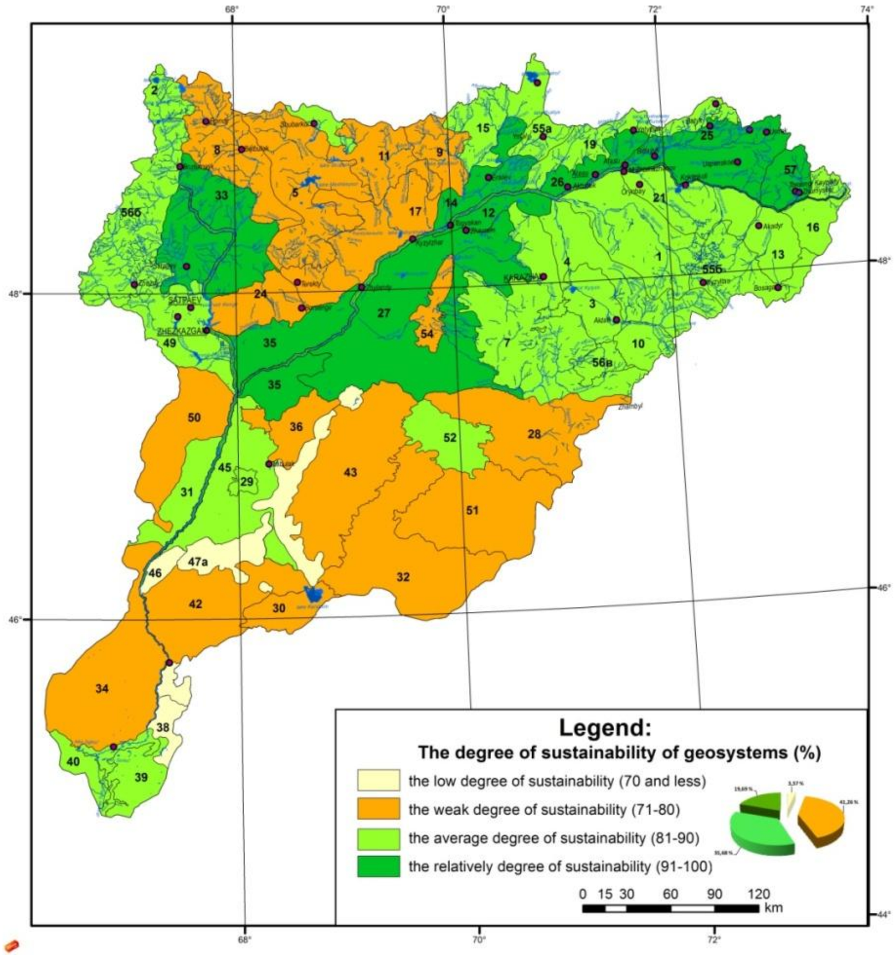

***Figure 2.** Map of landscape sustainability of Saursu Basin, Kazakhstan in conditions of human impact*

The last group of geosystems (landscapes 46–48) was determined like those with a low to anthropogenic impact. They are hilly-ridged Aeolian plains with wheatgrass, artemisia terrae-albae-ceratoides vegetation on the sand, and floodplain meadow soils. The geosystems mainly have an accumulative position with flat relief and slopes around 0–2°. Sandy hydromorphic soils have a desuctive-exudational water regime, moderately saline, and an alkaline reaction of the soil solution. The humus content in the layer of 0–20 cm is around 0.3–0.4%, humus horizon is around 5–7 cm. The cation exchange capacity is around 16–17 mg.-ekv/100 gr. soil. The canopy area is around 25–30%.

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In general, geosystems with a low degree of sustainability (41.26%) and an average degree of sustainability (35.68) dominate the study area. In the future, information about geosystems, obtained via the assessment of sustainability will be interpreted from the standpoint of commercial geography, to develop the optimum environmental management structure. This structure includes information about the status of the potential sustainability of geosystems with regard to human impact, and the level of anthropogenic load on geosystems. Sustainability is regarded as a special natural resource because it an environmental assimilation capacity environment concerning the emission of substances and energy. Moreover, it can be used as an indicator to determine environmental policy during human impacts. It is assumed that the optimum environmental management structure does not lead to negative consequences, and does not reduce geosystems' features of resource and environmental forming. On the other hand, imperfect environmental management formed without the landscape features of the territory leads to disruption and degradation.

Conclusions

The Sarysu Basin region is a closed drainage basin with poor biodiversity eroded by aquatic, but also the strength of intensive and direct air links. The Basin is located mainly within desert and semi-desert landscape zones. The Basin's geosystems have a combination of natural factors, which strengthen the pollution of geosystems. Hence it cuts down the centralization, streamlining, self-organization and sustainability of the landscape.

The integral evaluation of the level of sustainability of natural complexes to anthropogenic impacts by identifying landscape factors of the geosystems allows the identification of landscapes with different levels of sustainability potential (from relatively stable to weakly stable). It demands different approaches in the development of environmental management structures. The research identified geosystems with sustainability relative to human impact located at the Sarysu flow in the upper steam. The upper steam geosystems are eluvial and have favourable thermal and hydrothermal conditions, whereas geosystems located at the flow subsidence are accumulative, with low resilience.

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EVALUATION OF GROUNDWATER QUALITY AT COCONUT HUSK RETTING AREA

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Abstract. This paper evaluates the quality of the groundwater in and around a coconut husk retting area. The area selected for the study is Mondaikadu of Kanyakumari district, Tamil Nadu, India, which is very near to the coast of the Indian Ocean. Groundwater samples were collected during summer, winter and pre-monsoon season and analyzed using analytical instruments. It was observed that the groundwater quality is significantly affected by coconut husk retting process. The pH level in the groundwater was found to be unacceptable because it was below the permissible limit of 6.5 to 9.2 as per the World Health Organization (WHO) and Bureau of Indian Standards (BIS). All other parameters such as Total Dissolved Solids (TDS), Electrical conductivity (EC), Hardness, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Ferrous (Fe), Manganese (Mn), Nitrate (NH_3), Chloride (Cl), Fluoride (F), Sulphate (SO_4) are within the limits. Low pH water was neutralized using addition of Sodium Hydroxide methods (NaoH), addition of soda ash and aeration process. Using correlation analysis, the dependent and independent variables were selected and regression analysis was conducted for these variables and the best fit regression model was created.

Keywords: *correlation, regression, pH, electrical conductivity, neutralizer*

Introduction

Water is one of the most common and most precious resources of our earth. About 80% of the earth's surface is covered with water. It cannot be made electronically or hydrologically or by any other means (Brindha and Elango, 2011). It may be classified as groundwater and surface water. Groundwater is the main source for drinking purpose. Groundwater contains less pollution as compared to surface water and does not have suspended solid particles also having less turbidity (Gajendran et al., 2013). The most important environmental issue is groundwater contamination (Oyelami et al., 2013). About 70 % of India's groundwater resource has been contaminated by organic, inorganic and biological pollution (Anitha and Sugirtha, 2013). Most of the contamination occurs on account of mining, land clearance, agriculture, acid precipitation, domestic and industrial waste (Appelo and Dieke, 2005). Population growth and rapid urbanization are one of the reasons for qualitative degradation of groundwater (Abd El-Salam and Abu-Zuid, 2015). The soil influence on the water quality of an area is very important and it can be described as the process controlling the exchange of chemicals between the soil and water (Hesterberg, 1998). The groundwater quality is constantly changing with respect to season and climate. As per WHO, about

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80 % of all the diseases in human beings is caused by impure water. Recent results indicated that many groundwater quality analyses have been conducted in different areas, such as landfill leachate, limestone aquifer, municipal land fill lagoons, rural settlement, municipal solid waste site etc. (Abd El-Salam and Abu-Zuid, 2015; Jamshidzadeh and Mirbagheri, 2011; Longe and Balogun, 2010; Adekunle et al., 2007; Kurakalva et al., 2016). It is important to identify the source of pollution to avoid future degradation of groundwater quality (Dieng et al., 2017). This is the first study to conduct the groundwater quality analysis near the coconut husk retting area.

In the process of retting of a coconut husk lot of solid waste is produced. This gets mixed up with solids and gradually decomposes. This merges with the soils and the aquifer and pollutes the groundwater system. The effluent generated from the coir industry is acidic, also contains phenolic compounds and other toxic substances (Simple Lotus, 2012). The heavy pollution caused by the retting yards is found to have serious environmental degradation and ecosystem damage. Many researchers analysed the physic- chemical characters of the retting water and finally they found that it will affect the retting water and land. It causes serious health impacts to the people (Najee and Philipose, 2013; Manoj, 2014). Due to the contamination of surface water the soil may get contaminated (MaryHelen et al., 2011). This leads to groundwater contamination. In order to assess the quality of water continuously, prediction is needed. The prediction can be used for resource planning and management if it is of acceptable accuracy (Seyyed et al., 2013). Recently, mathematical, statistical, computational and spatial analysis is used to simulate and assess many aquifer water quality parameters. Spatial analysis of pollution is used to identify the source of the pollution in a particular study area (Ang et al., 2016).

Regression analysis was the linear modelling tool used for prediction. It is a form of predictive modelling technique which investigates the relationship between a dependent and independent variable**.** This technique was used for forecasting, time series modelling and finding the causal effect relationship between the variables. The curve must be fitted to the data points, in such a manner that the differences between the distances of data points from the curve or line was minimized. In this study it was used to predict one dependent variable by using other independent variables.

The main aim of this study is to assess the groundwater contamination in the area specified, to compare the water quality parameters with drinking water quality standards, suggestions were given to treat the contaminated water and the groundwater quality modelling is created. Regression analysis is used to create the groundwater models. The parameters selected for analysis were based on the correlation matrices.

Kanyakumari District is mostly covered by costal area. The geographical extent of Kanyakumari District is 1,672 km^2. The soil type is mostly red loam and laterite with coastal alluvium in the south. The district has a warm and humid climate with a maximum day temperature ranging between 24 °C and 34 °C throughout the year and an annual rainfall of 146 cm. The main occupations of the coastal area are coir making and fishing (ENVIS Centre, Tamil Nadu). In the coir production process, the coconut husk is soaked in a retting pond for a period of 6 months to one year. During this period, it may produce a lot of organic, inorganic and biological components. This process is highly affecting the quality of water in the retting pond. It produces a bad smell around the surrounding places.

The area taken for this study is Mondaikadu at Kanyakumari District. Mondaikadu lies between 8°9’47” latitude and 77°16’48.09” longitude. It is a Panchayat town of

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Kanyakumari District, Tamil Nadu. Here, the coconut husk retting operation is carried out near the AVM canal. That canal water is used for the coconut husk retting process.

Material and methods

The water sampling process was done during winter, summer and monsoon seasons for the period of three years from December 2012 to November 2015. There were 10 groundwater samples collected from different bore wells near the retting site. The specifications of the different bore wells are mentioned in *Table 1*. Samples were collected in one 1itre polythene bottles, which were pre cleaned with concentrated hydrochloric acid and distilled water. pH was measured on the site with a digital pH meter (Ahamefula et al., 2013). This was done to avoid unpredictable changes in characteristics as per the standard procedure (APHA, 1998). All the collected samples were preserved in the refrigerator and the analysis such as TDS, EC, Hardness, Ca, Mg, Na, K, Fe, Mn, NH3, Cl, F, SO_4 were completed in the Environmental engineering laboratory within two days.

Table 1. *Specifications of bore wells*

Sample no.	Latitude	Longitude	Elevation (m)
S1	8°9.678'N	77°16.724'E	49.3
S2	8°9.7'N	77°16.729'E	64.8
S3	8°9.665'N	77°16.753'E	37.2
S4	8°9.650'N	77°16.755'E	45.6
S5	8°9.617'N	77°16.866'E	78.2
S6	8°9.642'N	77°16.954'E	34.4
S7	8°9.579'N	77°16.071'E	51.7
S8	8°9.571'N	77°16.086'E	50.4
S9	8°9.589'N	77°16.032'E	86.1
S10	8°9.607'N	77°16.027'E	54.7

Results and discussion

Statistical analysis

Physical and chemical properties of the groundwater in all the three seasons are evaluated. Each season has different range of parameters. In general, the winter season has higher values compared to the other two seasons. As compared to WHO (1993) and BIS (2009) standards all parameters are within the permissible limit, except pH. *Table 2* shows the comparison of BIS, WHO standards and minimum, maximum range of each parameter.

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Table 2. *Statistics f the hydrogeochemical properties with WHO and BIS standard for winter season*

Parameter	Winter season		Summer season		Monsoon season		BIS standard	WHO standard
	Min.	Max.	Min.	Max.	Min.	Max.		
Turbidity	0	1	0	1	0	2	1-5	5
TDS	68	290	60	216	61	383	200-500	1000
EC	103	439	98	210	93	580	700-3000	-
pH	4.4	6.4	5.2	6.2	4.61	6.03	6.5-8.5	6.5-8.5
Hardness	30	100	18	56	26	164	300-600	500
Ca	8	26	6	21	7	16	75-200	75-200
Mg	2	10	1	4	2	15	30-100	50-150
Na	8	46	6	32	9	44	-	200
K	1	11	1	9	1	7	-	12
Fe	0	0.12	0	0	0	0	0.3	0.3
Mn	0	0	0	0	0	0	0.1-0.3	0.1
NH_3	0	0.08	0	0.46	0	1	0.5	1.5
Cl	20	96	20	86	20	164	250-1000	250
F	0	0.2	0	0.2	0	0	1-1.5	1.5
SO_4	3	17	3	17	3	16	200-400	250

pH value

The pH range of groundwater for all the three seasons is below the permissible level of WHO and BIS, which means the water is acidic and corrosive in nature. *Figure. 1* shows the spatial variation of the pH in various sampling sites for three seasons. The map was interpolated by using kriging method. Kriging is a linear interpolation procedure that provides linear unbiased estimation for quantities, which vary in space and it is an advanced geostatistical procedure for interpolation (Gunarathna, 2016).

The main reason for low pH is a low concentration of ions present in the groundwater. The effluent from the retting site is acidic, this may be the reason for low pH in the study area. As per WHO, if the pH range is less than 6.5 or greater than 9.2 it affects the portability of the drinking water. But it has no direct impact on human health (Jasem et al., 2010). U.S. environmental protection agency says that low pH in water can damage the metal pipe and give the sour taste (EPA, 2003). It may be treated by adding neutralizer to the water. Neutralizer prevents the water from reacting with the metal pipe.

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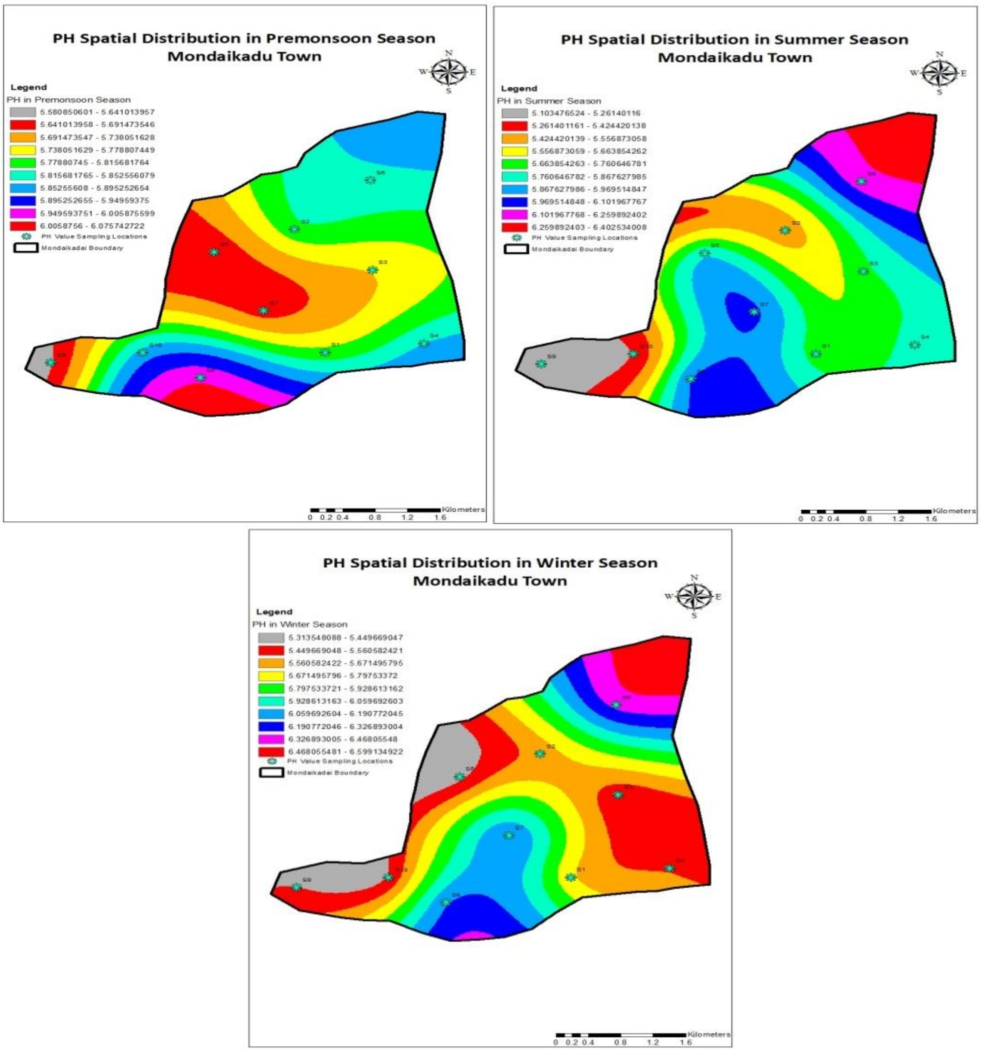

Figure 1. *Spatial variation of pH for three seasons*

Neutralize the low pH water

1. Addition of sodium hydroxide (NaOH)

This is the one of the best methods to increase the pH level in water. Titration method is used to determine the amount of NaOH needed to raise the pH of the water. NaOH solution is prepared by adding 4 g of NaOH pellets to 100 ml of distilled water. Then the NaOH solution obtained is taken as the burette solution and titrated against 20 ml of water sample. Phenolphthalein ($C_{20}H_{14}O_4$) is added as the indicator and the end point is colourless to pink. The burette reading gives the amount of NaOH used for 20 ml of the water sample. If sodium hydroxide is added manually, good ventilation should be maintained to avoid breathing vapours and wear protective gloves, goggles to avoid skin and eye contact (Wagenet et al., 1995).

The amount of NaOH added to the water is calculated using the below formula:

$$\text{NaOH needed} = \frac{(\text{NaOH used per litre of Sample} \times \text{Normality of NaOH} \times \text{Equivalent weight of 1 litre of 1 N NaOH})}{1000}$$

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The calculated quantity of NaOH added to the water (H_2O) and mixed thoroughly suddenly pH level is increased by the following reaction:

$$H_2O + NaOH \rightarrow \text{aqueous solution containing NaOH}$$

Water reacts with NaOH to form an aqueous solution containing NaOH.

2. Addition of soda ash

120 g of soda ash is added to 1 L of water (Glenda and Jennings, 1996). The sample is stirred well, so that the soda ash gets completely dissolved in water. Now the pH of the water sample is measured. The reaction is taking place as follows:

$$2H_2O + Na_2CO_3 \rightarrow 2NaOH + H_2CO_3$$
$$H_2CO_3 \rightarrow H_2O + CO_2 \uparrow$$

Water reacts with sodium carbonate (Na_2CO_3)to form NaOH and carbonic acid. Carbonic acid decomposes to water and carbon dioxide.

3. Aeration process

The process by which air is circulated through, mixed with or dissolved in a liquid is called aeration. In this method, water is taken in a vessel and air is supplied to the water using an aerator. Immediately air bubbles will be produced and the pH gets increased. Water displacement from the expulsion of bubbles can cause a mixing action to occur and the contact between water and the bubble will result in oxygen transfer. 3.78 L of water are aerated for 2 s to increase the pH of water to a permissible level (Kirby et al., 2007).

Table 3 shows the amount of pH present in the water before and after the neutralizing process. It seems that all methods of a neutralizer are very effective one.

Table 3. *pH before and after neutralizing*

Sample no.	Neutralizing method			
	Initial	NaOH	Soda ash	Aeration
1	5.98	7.95	7.69	8.14
2	5.81	6.83	8.05	7.86
3	4.67	8.07	7.70	7.74
4	5.26	7.66	6.99	7.17
5	5.32	7.48	8.22	8.32
6	4.74	7.52	8.12	7.58
7	4.33	6.98	6.89	7.71
8	4.60	7.31	6.91	7.35
9	4.4	7.1	6.7	7.12
10	4.61	7.3	6.86	7.31

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Correlation analysis

Correlation analysis is used to check the relation between the variables. It is the one of the methods of multivariate statistical study. Many statistical analysis were conducted to determine the temporal variations of the study area. Many statistical analysis were conducted in various research (Özdemir, 2016; Asfandyar et al., 2016; Tokatli, 2015). Correlation analysis is the easiest one among all.

All variables are used to check the correlation. The correlation coefficients are computed using the following equation (*Eq.1*):

$$r = \frac{\sum xy}{\sqrt{(\sum x^2 \mathrm{X} \sum y^2)}} \qquad \text{(Eq. 1)}$$

where $x = X - \bar{X}$; $x = \frac{\sum x}{n}$; $y = Y - \bar{Y}$; $y = \frac{\sum Y}{n}$; n = no of samples.

SPSS 20 software was used to conduct the correlation analysis. There were 50 samples used to find out the correlation between the variables. If the correlation coefficient (R) between the parameters are closer to 1 then the parameters are more positively correlated.

Table 4 shows the correlation matrix for the different variables. It shows that the correlation between fluoride (F) and Cl is having lower R value, that is 0.001.

Table 4. *Correlation matrix between the variables*

Parameter	Turbidity	TDS	EC	pH	Alkalinity	Hardness	Ca	Mg	Na	K	Cl
Turbidity	1										
DS	0.482	1									
EC	0.47	0.984	1								
pH	0.085	-0.321	-0.277	1							
Alkalinity	0.355	0.7	0.708	-0.253	1						
Hardness	0.501	0.926	0.951	-0.184	0.633	1					
Ca	0.291	0.734	0.706	-0.14	0.618	0.665	1				
Mg	0.464	0.882	0.909	-0.33	0.605	0.932	0.519	1			
Na	0.382	0.951	0.937	-0.319	0.715	0.817	0.779	0.772	1		
K	0.24	0.699	0.637	-0.344	0.612	0.447	0.665	0.422	0.803	1	
Cl	0.469	0.97	0.946	-0.28	0.557	0.923	0.667	0.861	0.889	0.607	1

The reason for the low correlation is due to the ionic alteration by anthropogenic activities (Bodrud-Doza et. al., 2016). EC is highly correlated with TDS, hardness, Mg, Na, Cl, and the values of correlation coefficient (R) are given in the *Table 5*. The reason

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for the high correlation was that the parameters are related to each other. If the EC was highly correlated parameter it means it depends on TDS, hardness, Mg, Na, Cl. If, these values increase it means EC also increases and if these values reduce that means EC value reduces. Therefore EC was called a dependent variable and other parameters are independent variables. These independent variables are not dependent on EC. These highly correlated variables were used as data for regression analysis.

Table 5. *Correlation coefficient (R) value of most correlated parameters*

Parameters	R value
EC-TDS	0.984
EC-Hardness	0.951
EC-Mg	0.909
EC-Na	0.937
EC- Cl	0.946

Regression model

The aim of the regression analysis is to determine the relation between the dependent variable and independent variable. The dependent variable is EC and the independent variables are TDS, Hardness, Mg, Na, and Cl are selected from the correlation analysis. Since the number of independent variables is more therefore multiple linear equation is created. *Eq. 2* shows the multiple linear equation of the variable:

$$y = a_1x_1 + a_2x_2 + a_3x_3 + a_4x_4 + a_5x_5 + c \quad \text{(Eq. 2)}$$

where y represents the EC content and, x_1, x_2, x_3, x_4, x_5, represent the TDS, Hardness, Mg, Na, Cl respectively; a_1, a_2, a_3, a_4, a_5 are the corresponding coefficient of the independent variables, c is the constant of the regression equation. The best fit model is found based on the coefficient of determination (R^2). If it is nearer to 1, there is a good relationship between the dependent and independent variables.

Multiple linear regression analysis were conducted in the Matlab programming for the regression model. The constants are calculated and the model is developed as *Eq. 3*:

$$y = 0.0129x_1 - 0.141x_2 + 0.112x_3 + 6.46x_4 + 1.64x_5 + 131.66 \quad \text{(Eq. 3)}$$

Figure 2 shows the regression plot for the target and predicted EC content. The R value of the regression model is 0.95995. It shows that there is a good relationship between the target and the output of the model. *Figure 3* shows the linear relationship between the observed and regression predicted EC. The coefficient of determination, R^2 value is 0.9215; this value shows that the predicted EC has a good coefficient of determination with observed EC.

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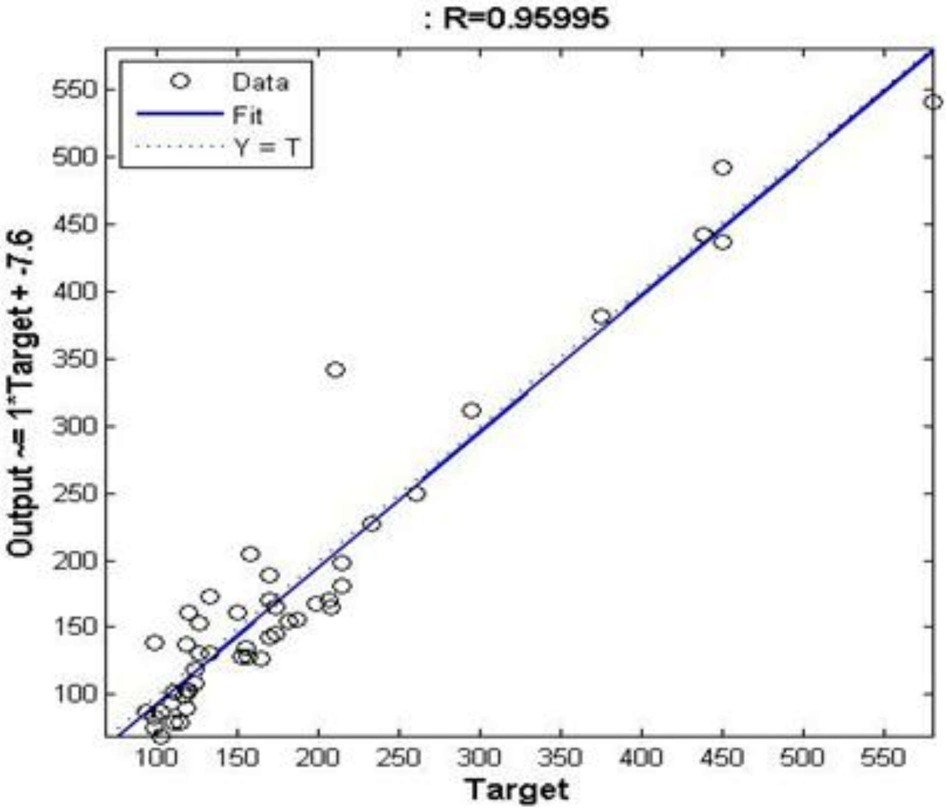

Figure 2. *Regression curve for input and target*

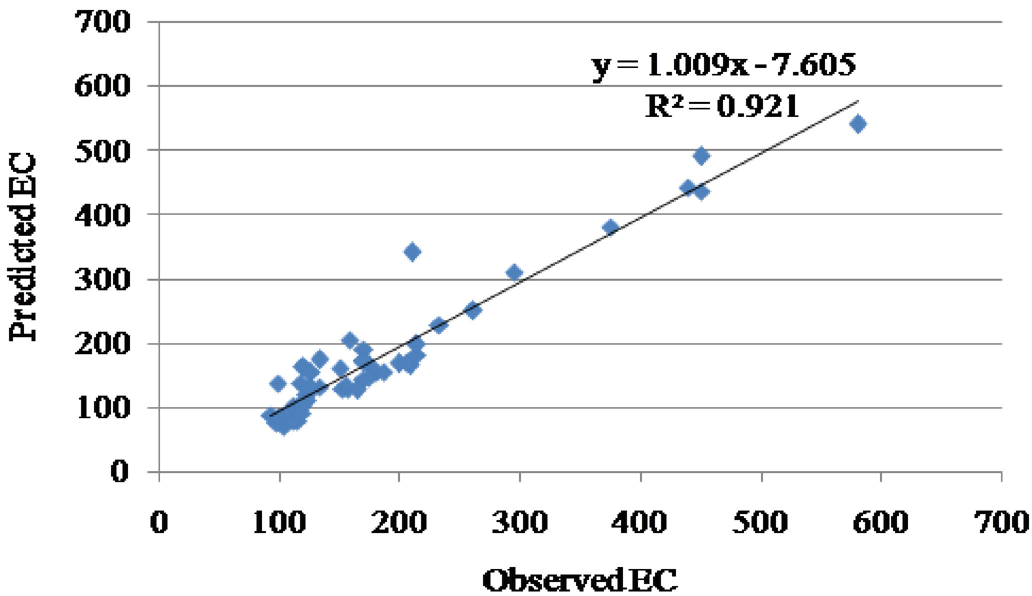

Figure 3. *Linear relation between observed and predicted EC*

From *Figure 4* it is clear that the observed EC was closer to predicted EC.

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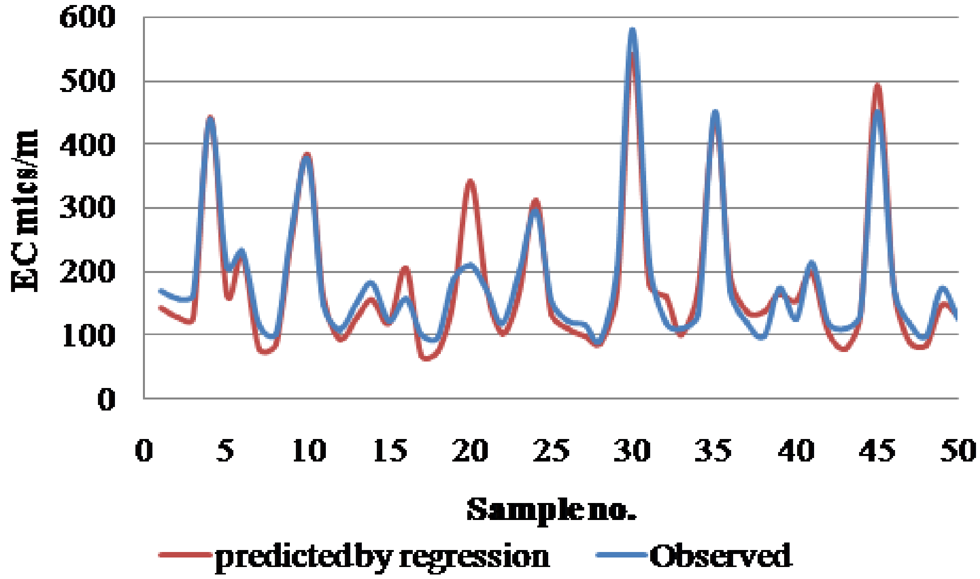

Figure 4. *Comparison of observed and predicted EC*

Conclusions

The result of study indicates that the borewell water in the coconut husk retting area are suitable for domestic purpose. The experimental analysis reveals that the parameters such as TDS, EC, Hardness, Ca, Mg, Na, K, Fe, Mn, NH_3, Cl, F and SO_4 of all 50 samples are within the prescribed limit of WHO and BIS, except for pH. The water can be used for drinking after neutralising by addition of NaOH, addition of soda ash and aeration methods.

The results of correlation analysis shows that EC was highly correlated with other parameters. The regression analysis shows the coefficient of determination for the observed and predicted EC was 0.9215, which was close to one, therefore regression model was a good prediction tool for this problem.

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AN INVESTIGATION ON RELEASING TREATED WILD ANIMALS INTO THE NATURE IN TURKEY

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Abstract. The purpose of this study was to evaluate the general profile of wildlife in Turkey in terms of injured wild animals, their treatment and re-introduction to the nature. It was analysed the records regarding the Treatment and Rehabilitation of wild animal, collected from 2012 to 2015 and determined the number of wild animals injured in nature and treated, the number reintroduced to nature, the amount settled in zoos and having died during this process. Of 11,110 treated animals, 50.18 % were reintroduced into nature, 24.95 % were settled into zoos and the remaining 24.87 % died. However, the evaluation in this study also showed that the number of both harmed and dead animals in nature has been gradually increasing by years ($p < 0.05$). This study covered a general evaluation of the damage on the wild animals in Turkey; therefore there is a need for detailed exploration of these damages, including the causes, consequences and proposals for solutions in the future. In this context, it is also important to increase opportunities for cooperation between Ministry of Forestry and Water Affairs, other countries, universities and relevant NGOs.
Keywords: *recording system, conservation, rehabilitation, wildlife damage*

Introduction

Turkey is a rich country in terms of the variety of species within its three bio-geographical zones, Europe-Siberia, Mediterranean and Iran-Turanian (Demirsoy, 2002; Eken et al., 2006; Atalay and Efe, 2015). As in other countries, however, industrialization and technological development, population growth, unplanned urbanization, thoughtless use of agricultural chemicals, environmental pollution and drying out of water sources, continues to cause serious habitat loss that threatens Turkey's wildlife (Hadidian et al., 2006; Ogurlu, 2008; Aslan et al., 2011; Akkuzu et al., 2015). Diseases, injuries and death in wild animals are mostly due to human activity and unnatural causes (Vitouse et al., 1997; Sanderson et al., 2002; Manfredo and Dayer, 2004; Thompson et al., 2010; Burton and Doblar, 2004). In many developing countries, with widespread poverty and weak institutionalization, intense pressures to hunt, and the conversion and fragmentation of wildlife territories cause conflicts between humans and wildlife (Bulte and Rondeau, 2007; Gore et al., 2008; Chynoweth et al., 2016; Ambarlı et al., 2016). Yet, wildlife is not only critical to maintaining the integrity of the earth's ecosystem but wild animals are also biological indicators of environmental conditions in urban and rural areas. Although public interest in wildlife has increased nowadays, wildlife species and habitats are more threatened than ever before. In

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response, the rehabilitation of wildlife has been highlighted in recent years, with studies being conducted with official rehabilitation centers on how to mitigate human impact on wildlife (Burton and Doblar, 2004). The determination of morbidity and mortality rates in wildlife is important for both wildlife conservation projects and resettlement or relocation projects (Gilmartin et al., 1993). As well as its importance for ecosystems, wildlife is also a significant risk element in the emergence of new zoonoses (Cabello and Cabello, 2008; Thompson et al., 2010; Rout et al., 2016). Approximately 75 % of emerging diseases during the past few decades have included zoonoses originating from wildlife (Bengis et al., 2004; Jones et al., 2008).

Responsibility for the protection, development and sustainable management of wildlife resources and biodiversity in Turkey lies with the Ministry of Forestry and Water Affairs. In Turkey, various laws have been enacted to protect, support and maintain wildlife for future generations, with various organizations for the conservation of nature being established and Turkey becoming a party to international conventions. Although new regulations prepared with a view to these conventions make an important contribution protecting wildlife, it is essential that Turkey continues to increase such efforts, and that the value of biological assets and their relevant rights are disseminated comprehensively (Aslım et al., 2012). The General Directorate, which is responsible for protecting wildlife resources and biological diversity, supporting them and preserving them for future generations, carries out its duties through 15 regional directorates under the General Directorate. Game and Wildlife Units within the regional directorates co-operate with Wildlife Rescue and Rehabilitation Centers established by the ministry, veterinary faculties and zoos, in accordance with protocols governing the protection, treatment and release of wild animals (www.ormansu.gov.tr).

This study is the first research to reveal the suppression on wildlife in Turkey and to draw attention to the damage in wild animals. Despite being a rich country in terms of wildlife, there is a big deficiency in Turkey in terms of the studies in this area. The sustainability of wildlife depends on the existence and continuity of such investigations. In this respect, the purpose of this study was to evaluate the general profile of wildlife in Turkey in terms of injured wild animals, their treatment and re-introduction to the nature and make recommendations.

Materials and Methods

The data used as the material of this study which covers the period between 2012 and 2015 was collected from the Department Directorate of Game and Wildlife of the Ministry of Forestry and Water Affairs (DKMPGM) in Turkey.

The research material comprises information forwarded to DKMPGM from the 15 regional directorates since 2012 while data for 2015 includes records to October. The records contain information regarding the number of animals seized for violation of Law 4915 (Official Gazette, Date: 11/7/2003, Number: 25,165) and the CITES convention (Convention on International Trade in Endangered Species of Wild Fauna and Flora – Accession: 23/09/1996, Entry: 22/12/1996), the number of animals harmed in nature and treated, the number of treated and released animals, and the number of animals placed into rescue centers or zoos. The number of dead animals was calculated from inventory records. Data regarding seized animals for violating Law 4915 and the CITES convention were eliminated. Data were analyzed by using SPSS 20.0 computer software package program. General analysis of data

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was carried out using frequency counts and percentages. Comparisons between the annual records of wild animals was performed by ANOVA and significance was tested by Duncan test.

Results and Discussion

The relevant inventory figures for wild animals registered by the DKMPGM between 2012 and 2015 is given in *Table 1* and shows the number of animals recorded yearly.

Table 1. *Numbers of suffering wild animals registered 2012-2015*

Year	Animals treated and released into nature[A]	Animals treated and placed in the rescue centers/zoos[B]	Dead animals[C]	Total animals harmed in nature and treated[A+B+C]
2012	821	408	451	1680
2013	1211	643	535	2389
2014	1763	830	734	3327
2015	1780	891	1043	3714
TOTAL	5575	2772	2763	11110

[A+B+C]Total animals harmed in nature and treated

The total number of animals registered over the four years categorized by registration criteria is given in *Figure 1* of 11,110 wild animals harmed in nature and treated within this four year period, 50.18 % were released back into nature, 24.95 % were placed in zoos and the remaining 24.87 % died. The proportion of animals treated and released in Turkey was 50.18 %, which is consistent with a study of RIAS in, Portugal, a wildlife rehabilitation center. In 2015, this centre received 1,335 wild animals including reptiles, birds and mammals. Of these 52.3 % was released in to nature after treatment and rehabilitation while 22 % deaths were determined (http://www.aldeia.org/portal/user/documentos/relatorio_RIAS_2015.pdf.).

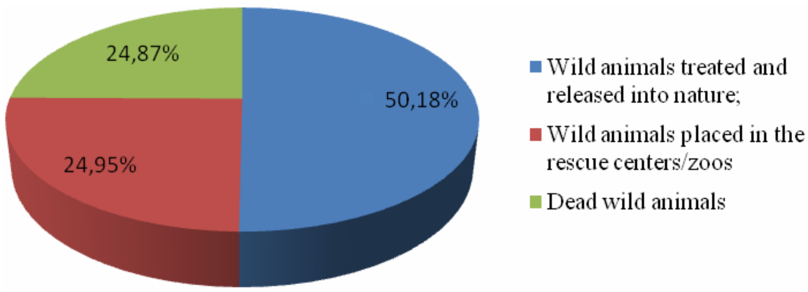

Figure 1. *The total distribution of wild animals registered during 2012-2015 (4 years)*

The distribution of recorded wildlife by taxonomic group shows that almost 85 % of registered species were birds and mammals (*Figure 2*). As *Figure 2* shows, according to the number of animals registered, the most frequently injured and/or diseased species in Turkey are birds. However, because there were no data about the causes of injury

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and/or disease, the researcher could not compare and/or analyse these issues in the study. Records are also available, in fewer numbers than for birds or mammals, for invertebrates, fish, amphibians and reptiles. Due to the seasonal changes concomitant with its geographical location, many species use Turkey as a feeding area or overwintering and breeding area (Baran, 2005; Kiziroğlu, 2008). Furthermore, Anatolia is also a transit zone during spring and autumn bird migrations, with many species migrating across Turkey between Europe, Africa and Asia (Hagemeijer and Mundkur, 2006; Üner et al., 2010; Şekercioğlu et al., 2011).

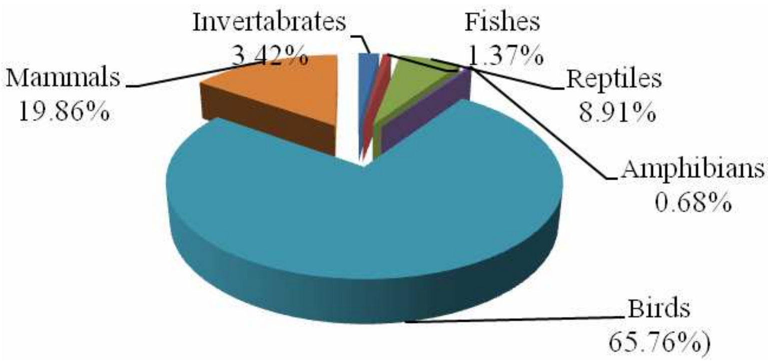

Figure 2. *The taxonomic distribution of recorded wild animals*

The categorization of the recorded wild animals by loss and compensation status is evaluated in *Table 2*. It clearly shows that number of wild animals that were harmed and died in nature. However, the number of wild animals released or placed into zoos has not changed.

Table 2. *Changes in recorded damaged wild animals by year*

Years	Animals treated and released into nature^{A}	Animals treated and placed in the rescue centers/zoos^{B}	Dead animals^{C}	Total animals harmed in nature and treated^{A+B+C}
	Mean±SE	Mean±SE	Mean±SE	Mean±SE
2012	54.73±7.54	27.20±7.74	30.07±8.99^{b}	112.00±17.84^{c}
2013	80.73±15.17	31.07±17.37	35.67±7.48^{b}	159.27±22.39^{b}
2014	117.53±24.40	55.33±11.69	48.93±11.37^{ab}	221.80± 38.48^{b}
2015	118.80±31.47	68.07±20.52	69.53±12.46^{a}	256.40±56.94^{a}
P value	0.12	0.19	0.04*	0.04*

$^{A+B+C}$Total animals harmed in nature and treated
*Means in the columns followed by different letters indicate statistically significant differences ($P < 0.05$)

According to *Table 2* while this study found no difference in the data between numbers of animals released into nature or placed into zoos while there were significant ($p < 0.05$) increases in both total animals harmed in nature and treated and both failed to respond to treatment and subsequently dead animals. The reason for this statistical increase in the number of animals dying by years may be related to the increase in the number of animals damaged in the nature by years. The increase in the

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number of animals damaged in the nature may be due to human activity and unnatural causes by years (Ogurlu, 2008; Aslan et al., 2011; Akkuzu et al., 2015).

The reason for the increase in the number of dead wild animals in recent years may be resulted from both the education and implementation studies on the treatment and rehabilitation of wild animals in Turkey have started relatively recently compared with many countries and the numbers of wildlife rescue and rehabilitation centers and the numbers of veterinarians and wildlife experts are inadequate. Although it seems ironically, the recent increase in the number of harmed and deaths in wild animal might have been due to the increase of the result of conservation and monitoring activities, scientific projects, social awareness and training activities of DKMPGM in cooperation with universities and NGOs; by this way it may be easier to access and record than in the past.

Conclusion

Consequently, in this research it was found that the damage of wild animals was increasing from by years. This situation shows that wild animals are under some threats in Turkey. These threats on wildlife should be identified and produced solutions. The decisions taken by DKMPGM to establish a Wildlife Rescue and Rehabilitation Center in each region promises hope for the future of Turkey wildlife. Additionally, increasing the number of wildlife rescue and rehabilitation centers, providing opportunities for the training of wildlife veterinarians and rehabilitators, supporting wildlife researches, dissemination conservation and awareness-raising activities are important for sustainable wildlife in Turkey. In this context, it is also important to increase opportunities for cooperation among the government ministry, other countries, universities and relevant NGOs. This study is the first research about the Turkey wildlife damage. Following this first study, which investigates the suppression of wild animals in Turkey, there is a need for studies that include causes, consequences and suggestions for solutions of casualties on wild animals in Turkey. These studies are important not only for the wildlife of Turkey, but also for the sustainability of the wild life which is the common ecological heritage of the whole world.

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THE INFLUENCE OF PLANT GROWTH SATGE, INDIVIDUALS OF SPECIES, AND EXTRACTION METHODS ON THE ESSENTIAL OIL CONTENT AND THE CHEMICAL COMPOSITION OF *PRANGOS FERULACEA* (L.) LINDL

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Abstract. *Prangos ferulacea* is one of the species of essential oil plants, which is appreciated because of its value in medicine, perfumery, and forage industries. In order to gain the optimum oil yield of *P. ferulacea*, the essential oils of fertile and infertile individuals were isolated from the aerial parts by steam distillation and hydrodistillation methods at three maturity stages of pre-flowering, flowering, and seeding. The oils were analyzed by capillary GC (Gas Chromatography) and GC/MS (Gas Chromatography/Mass Spectroscopy). The extraction yields from the aerial parts of *P. ferulacea* were found to be 0.17% and 0.24-0.29% for steam distillation and hydrodistillation, respectively. The highest oil yield was obtained at the flowering stage in infertile individuals (0.29% w/w) for hydrodistillation. Thirty-four components, comprising 99.98% of the total oil, were identified at the flowering stage of infertile individuals, in which (E)-caryophyllene (48.21%), α-humulene (10.28%), spathulenol (9.36%), linalool (3.46%), and δ-3-carene (3.37%) were recognized as the major components. Based on our findings, essential oil yields vary considerably from stage-to-stage and are also influenced by extraction methods. As well as, we found that infertile individual at the flowering stage can strongly enhance the quality of commercial oils in *P. ferulacea*.
Keywords: *Prangos ferulacea, steam distillation, hydrodistillation, (E)-Caryophyllene, α-Humulene, δ-3-Carene*

Introduction

The genus *Prangos* consists of 30 species, 15 of which grow wild in many regions of Iran, and five are endemic (Mozaffarian, 1996). *Prangos ferulacea* (L.) Lindl. is found in the Balkans, Italy, Sicily, W. Syria, Caucasia, Turkey, and semi-arid regions of Iran (Rechinger, 1987; Ghahreman, 1997). This species is well-known for its economic importance in the form of various essential oils and for its high forage quality (Ayres et al., 1994; Sefidkon et al., 1998; Razavi, 2012).

The storage of essential oils in *P. ferulacea* is not restricted to specific parts of the plant. In fact, essential oils occur in roots, stems, leaves, flowers and seeds, or in the plant as a whole. Various compounds have been already identified in the oil of *P. ferulacea*. The number of components in the aerial parts and the seed is more than 30

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components according to Sefidkon and her colleagues (1998) and Razavi (2012). Moreover, 39 and 33 components were reported in the fruits (Massumi et al., 2007) and the roots (Sajjadi et al., 2011), respectively. Besides the applications in cosmetics, perfumes, and flavors, essential oils have been studied with regard to their antimicrobial properties (Eshbakova et al., 2006; Massumi et al., 2007), antioxidant properties (Razavi, 2012), hypoglycemic activities (Soltani band et al., 2011; Mohammadi and Zare, 2013), and antihyperlipidemic effects (Ramesh and Pugalendi, 2005).

Previous phytochemical studies on *P. ferulacea* (L.) Lindl. have indicated the presence of coumarin, alkaloid, flavonoid, and terpenoid derivatives. The performed investigations have reported the principal components in the leaves as follows: α-pinene (28.2%), δ-3-carene (15.3%), limonene (8.1%), and myrcene (6.7%); and in the fruit oil: α-pinene (25.4%), 3-n-butylphthalide (13.8%), limonene (10.6%), δ-3-carene (9.1%), and sabinene (8.1%); and in the roots, δ-3-carene (22.5%), β-phellandrene (11.8%), α-pinene (8.6%), terpinolene (7.2%), p-cymene (6.3%), α-phellandrene (6.2%), and myrcene (4.5%).

Not only the ontogenesis, but also the intraspecific variation in particular (i.e., the differences between the individuals of an identical species), must be taken into account for the appropriate treatment of the plant samples, in order to determine the quality and variability of essential oil with regard to their composition. In general, *P. ferulacea* individuals annually seed 5 to 15 percent (Gheitori et al., 1997), and the biological properties of *P. ferulacea* show that it is polycarpic perennial (polycarpic plants flower and fruit more than once in their lifetimes) (Reuther, 2013). Thus, this species has both fertile and infertile individuals at different ranges.

It is worth stating that even though essential oils may be produced from an endemic population, but physiological and environmental factors as well as extraction methods may play an important role in the essential oil quality, purity, and origin, and also the composition of aromatic plants (Hay, 1993; Hay and Svoboda, 1993; Jordán et al., 2013; Sellami et al., 2012). The economic importance of *P. ferulacea* organic compounds in perfumery, food, medicine, and pharmaceutical industries, depends on the extract of the oil during the plant growth stages, which can consequently lead to increase the yield, quality, and purity of components (Ayres et al., 1994; Sefidkon et al., 1998; Coskun et al., 2004; Razavi, 2012).

Hydrodistillation and steam distillation methods can obtain essential oils from the plant material and induce thermal degradation, hydrolysis, and water solubilization of some fragrance constituents or solvent residues (Faborode, 1996). As a part of the biochemical studies at the Plant Biocentre, both techniques have been simultaneously utilized in the volatile analysis of essential oils in order to develop appropriate methods for different purposes.

The aim of the present work is the investigation of the effects of different maturity stages and types of individuals on the essential oil content and the chemical composition of *Prangos ferulacea* (L.) Lindl. For comparison purposes, the essential oils obtained by hydrodistillation and steam distillation were used. To the best of the authors' knowledge, no report has yet been accomplished on the comparison of the essential oil contents and the chemical components of fertile and infertile plants in *P. ferulacea.*

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Materials and methods

Study Area

This study was conducted in the Bistoon mountains of Kermanshah province in Iran (north-eastern slope at 34°27′ N and 46°55′ E). The average annual temperature and annual rainfall are 11.88 ºc and 650 mm respectively that more precipitation is snow. At altitudes from 2200 to 2900 m. the plant grows. The site soil is classified as a Regosolic. In the upper 5 cm of the site soil below the litter layer, total soil nitrogen, total soil phosphorus and potassium per unit soil volume are 0.46 (%), 37.6 (p.p.m) and 610 (p.p.m) respectively.

Plant materials

During the annual growth, fertile and infertile plants grow together. First, at the pre-flowering stage, the aerial parts of fertile and infertile plants were cut on April, 12^{th} (stage 1). Then, at the flowering stage, the flowers appeared 42 days after leaf emergence on May, 6^{th} (stage 2). Finally, at the seeding stage, the seeds appeared 62 days after leaf emergence on June, 1^{st} (stage 3). They were cut regularly during May and June 2014 in a sample area of 50 m^2 and randomly replicated three times. At each maturity stage, 36 plant samples were harvested at 10 cm above the ground level. The samples were then dried without conditioning and grounded in a laboratory mill, until they passed through a 1-mm-pore-size screen for chemical analysis.

Hydrodistillation (HD) and steam distillation (SD)

The dried plant materials (including fertile and infertile individuals) were submitted by hydrodistillation and using a Clevenger-type apparatus at each stage of the plant growth (i.e., the pre-flowering, flowering, and seeding stages). Distillation was performed by using approximately 100 g of each plant sample for 2 hours. In order to determine the composition of the oil and the contents at the flowering stage for infertile individuals, the aerial parts of the plant were subjected to hydrodistillation and steam distillation methods. The volatile distillates were collected over anhydrous sodium sulphate and stored in the refrigerator at 4 °C before the analysis. The oil yield was calculated as v/w of the dried plant material. Overall, three oil samples (3 replicates) were prepared from each type of plant, as described above.

GC and GC/MS analyses

GC analyses were performed using a Shimadzu GC- 9A gas chromatograph equipped with a FID and a DB-1 fused silica column (60 m· 0.25 mm i.d., film thickness 0.25 lm). Oven temperature was programmed to 50 C for 5 min, and then increased to 250 C at a rate of 4 C/ min. Injector and detector temperatures were 250 and 265 C, respectively. The carrier gas, helium, was adjusted to a linear velocity of 30 cm/s. The SFE samples (1 ll) were injected into the GC (without any further dilution) using the split mode with a split ratio of 1/60. Hydrodistilled extracts were diluted 30 times and 1 ll of diluted solution was injected into the GC with the same split ratio. The GC/MS analysis was carried out on a Varian 3400 equipped with a DB-1 column with the same characteristics as the one used in GC. The transfer line temperature was 260 C. The ionization energy was 70 eV with a scan time of 1 s and mass range of 40–300 amu. The percentages of compounds were calculated by the area normalization method,

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without considering response factors. The components of oil were identified by comparison of their mass spectra with those assembled via a Wiley 5 mass spectra computer library or with authentic compounds. Data obtained were confirmed by comparison of their retention indices, either with those of authentic compounds or with the data published in the literature (Adams, 2007).

Statistical analysis

The data on the composition of oils were calculated by analysis of variance (ANOVA) using SPSS software (version 19) (1993).

Results and discussion

Essential oils normally contain a complex mixture of organic compounds. They are largely composed of a range of saturated or partly unsaturated cyclic and linear molecules of relatively low molecular mass and within this range, a variety of hydrocarbons and oxygenated compounds occur. Various parameters, such as environmental and experimental conditions, physiological and ecological responses, growth rates, and productivity, potentially influence on the content and composition of oils. In this study, the main focus has been on the potential of extraction methods, the type of individuals, and the stage of maturity.

In total, thirty-four components were identified in the essential oil of *P. ferulacea* at the flowering stage for infertile individuals, representing 99.98% of the oil. The main components were (E)-caryophyllene (48.21%), α-humulene (10.28%), spathulenol (6.73%), α-bisabolol (4.25%), and δ-3-Carene (3.37%) (*Table 1*).

Table 1. *The percentage composition of the essential oil obtained by hydro- distillation from Prangos ferulaceae (L.) Lindl at flowering stage*

No.		Compound	RI	(%) Composition	Mode of identification
1	*α*-Thujene		926	0.84	RI, MS
2	*α*- Pinene		949	Nd	RI, MS
3	Sabinene		974	0.72	RI, MS
4	Myrcene		999	1.19	RI, MS
5	*p*-Cymene		1024	2.39	RI, MS
6	Limonene		1028	2.18	RI, MS
7	δ-3-Carene		1030	3.37	RI, MS
8	1,8-Cineole		1035	Nd	RI, MS
9	γ-terpinene		1075	Nd	RI, MS
10	Terpinolene		1081	0.61	RI, MS
11	Dehydro linalool		1092	1.29	RI, MS
12	Linalool		1097	3.46	RI, MS
13	Octen-3-yl-acetate		1114	0.83	RI, MS
14	Myrcenol		1121	1.16	RI, MS
15	3-Octanol acetate		1122	1.01	RI, MS
16	-1-Terpinoel		1133	0.33	RI, MS

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17	p-Cymene-8-ol		1183	0.45	RI, MS
18	*cis*-pinocarvyl acetate		1313	0.34	RI, MS
19	*α*-longipinene		1353	0.82	RI, MS
20	Nevyl acetate		1364	0.68	RI, MS
21	Italicene		1405	0.5	RI, MS
22	E-Caryophyllene		1420	48.21	RI, MS
23	α-Humulene		1450	10.28	RI, MS
24	E-β-Farnesene		1458	3.21	RI, MS
25	7-epi-1,2-dehydro sesquicineol		1471	0.72	RI, MS
26	γ-Muurolene		1480	1.30	RI, MS
27	Epi- cubenol		1493	0.39	RI, MS
28	α-selinene		1497	0.78	RI, MS
29	Spathulenol		1577	6.73	RI, MS
30	Caryophyllen oxide		1582	0.41	RI, MS
31	globulol		1586	0.80	RI, MS
32	β-eudesmol		1650	0.73	RI, MS
33	α-bisabolol		1684	4.25	RI, MS
34	β -Bisabolene		1790	Nd	RI, MS

Nd: Not detected, or amount of component is below 0.1%.

Table 2 lists the yields, the growth stages, the fertility and infertility states, the compounds, and the chemical composition of the essential oil from the aerial parts of *P. ferulacea*, extracted by hydrodistillation. The highest oil yield was obtained at the flowering stage in the infertile individuals (0.29% w/w) by hydrodistillation.

In some essential oil compounds, significant differences were observed in the growth stage and in the type of individual treatments. Duncan's test was carried out to determine the composition parameters and means ± standard error and also, to test the impact of the growth stage and the type of individual treatments on oil compounds (*Tables 2* and *3*). Furthermore, *Figure 1* compared the percentage of the major components of *P. ferulacea* oils, extracted at different growth stages.

As can be seen in *Table 2*, some oil compositions of *P. ferulacea* were significantly affected ($p < 0.01$) by the type of individuals at the flowering and seeding stages. At the flowering stage, a significant difference was observed between fertile and infertile plants for octen-3-yl acetate, myrcenol, 3-octanol acetate, 1-terpineol, cis-pinocarvyl acetate, and β-eudesmol; and also, at the seeding stage, for α-thujene, myrcene, limonene, δ-3-carene, γ-terpinene, italicene, (E)-caryophyllene, (E)-β-farnesene, and caryophyllene oxide (*Table 2*). This difference may have been resulted from the existence of flowers and seeds in fertile plants. Akhgar et al. (2011) showed that the essential oil content and the chemical composition of *P. ferulacea* leaves is higher than fruits.

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Table 2. *Oils component of Prangos ferulaceae (L.) Lindl at different growth stages and extraction methods*

Compound	Before flowering	Flowering stage				Seeding stage			
		Fertile	Infertile	Mean±SE	Significance	Fertile	Infertile	Mean±SE	Significance
α-Thujene	-	1.1	0.84	0.97±0.12	ns	1.23	0.48	0.85±2.83	*
Sabinene	0.53	0.73	0.72	0.72±0.03	ns	1.44	1.09	1.26±0.15	ns
Myrcene	0.44	0.97	1.19	1.08±0.37	ns	1.08	0	0.54±0.43	*
p-Cymene	3.43	3.31	2.39	2.85±0.27	ns	-	-	-	-
Limonene	1.76	2.77	2.18	2.47±0.29	ns	4.33	1.88	3.10±0.72	*
δ-3-Carene	0.87	3	3.37	3.18±0.30	ns	6.82	1.44	4.13±2.03	*
γ-terpinene	-	-	-	-	-	0.96	0.63	0.79±0.15	*
Terpinolene	-	0.7	0.61	0.65±0.16	ns	1.05	1	1.02±0.28	ns
Dehydro linalool	-	1.45	1.29	1.37±0.16	ns	1.74	1.4	1.57±0.44	ns
Linalool	1.30	3.18	3.46	3.32±0.67	ns	0.35	0.31	0.33±0.03	ns
Octen-3-yl-acetate	-	0.21	0.83	0.52±0.18	*	-	-	-	-
Myrcenol	-	0.24	1.16	0.7±0.27	*	-	-	-	-
3-Octanol acetate	-	0.21	1.01	0.61±0.25	*	-	-	-	-
-1-Terpinoel	-	0.8	0.33	0.56±0.17	*	-	-	-	-
p-Cymene-8-ol	-	0.53	0.45	0.49±0.09	ns	1.77	0.87	1.32±0.40	*
cis-pinocarvyl acetate	-	0	0.34	0.17±0.16	*	0.27	0.13	0.20±0.04	ns
α-longipinene	0.22	0.74	0.82	0.78±0.20	ns	0.28	0.14	0.21±0.04	ns
Nevyl acetate	-	0.68	0.68	0.68±0.12	ns	0.69	0.54	0.61±0.06	ns
Italicene	-	0.66	0.5	0.58±0.05	ns	0.9	0.3	0.60±0.18	*
E-Caryophyllene	33.13	42.49	48.21	45.35±2.58	ns	27.05	32.13	29.59±5.36	*
α-Humulene	8.34	8.87	10.28	9.57±0.49	ns	3.34	2.91	3.12±0.36	ns

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E-β-Farnesene	2.48	2.79	3.21	3.0±0.17	ns	5.77	12.99	9.38±2.82	*
7-epi-1,2-dehydro sesquicineol	0.41	0.68	0.72	0.70±0.04	ns	0.76	0.57	0.66±0.06	ns
γ-Muurolene	-	1.49	1.30	1.39±0.12	ns	0.24	0.95	0.59±0.29	ns
Epi- cubenol	-	0.45	0.39	0.42±0.03	ns	-	-	-	-
α-selinene	0.73	1.14	0.78	0.96±0.13	ns	-	-	-	-
Spathulenol	1.39	9.36	6.73	8.04±1.05	ns	32.87	35.54	34.20±4.24	ns
Caryophyllen oxide	24.71	0.47	0.41	0.44±0.06	ns	2.94	1.59	2.26±0.97	*
globulol	1.16	0.91	0.80	0.85±0.13	ns	2.2	2.29	2.24±0.25	ns
β-eudesmol	1.84	0.55	0.73	0.64±0.06	*	-	-	-	-
α-bisabolol	1.37	4.36	4.25	4.30±0.68	ns	-	-	-	-
1,8-Cineole	1.02	-	-	-	-	-	-	-	-
α -Pinene	0.55	-	-	-	-	-	-	-	-
β -Bisabolene	1.01	-	-	-	-	-	-	-	-
Total		94.84	99.98			98.08	99.18		
Oil yield (% w/w)	0.26	0.22	0.29	-	-	0.22	0.24	-	-

Mean±SE: (Mean ± Std. Error), *P < 0.01, ns: not significant

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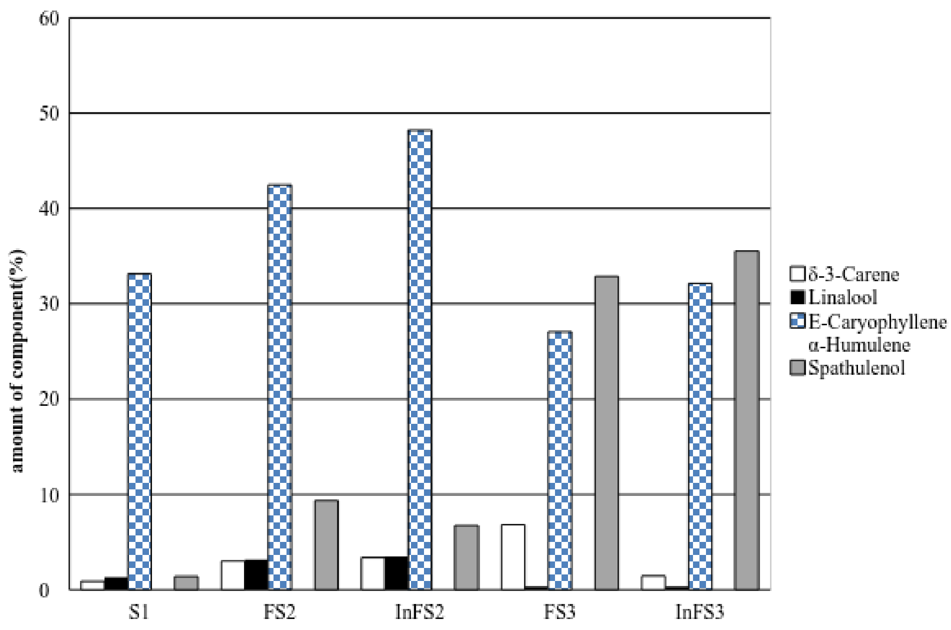

Figure 1. *Comparison between amounts of the major components (%) of P. ferulacea oils extracted at different growth stages. S1: before flowering (stage 1), FS2: the fertile plant in stage 2 or flowering, FS3: the fertile plant in stage 2 or seeding, InFS2: the infertile plant in stage 1, InFS2: the infertile plant in stage 2.*

Table 3 revealed that in 26 components of fertile and 23 compounds of infertile individuals at two growth stages (flowering and seeding), significant differences have been observed. This finding indicates that the phenology factor can play an important role in determining the composition of oils.

Table 3. *The chemical compositions (%) of P. ferulacea (Mean ± Std. Error) at flowering and seeding stages for fertile and infertile plants*

Compound	$Fertile^F$	$Fertile^S$	Significance	$Infertile^F$	$Infertile^S$	Significance
α-Thujene	1.10	10.23	*	0.84	0.48	ns
Sabinene	0.73	1.44	*	0.72	1.09	*
Myrcene	0.97	1.08	ns	1.29	0	*
p-Cymene	3.31	-	*	2.39	-	*
Limonene	2.77	4.33	*	2.18	1.88	ns
δ-3-Carene	3.00	6.82	*	3.37	1.44	ns
γ-terpinene	-	0.96	*	-	0.63	*
Terpinolene	0.70	1.05	ns	0.61	1.00	ns
Dehydro linalool	1.45	1.74	ns	1.29	1.40	ns
Linalool	3.18	0.35	*	3.76	0.31	*
Octen-3-yl-acetate	0.21	-	*	0.83	-	*
Myrcenol	0.24	-	ns	1.16	-	*
3-Octanol acetate	0.21	-	ns	1.01	-	*
-1-Terpinoel	0.80	-	*	0.33	-	*

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p-Cymene-8-ol	0.53	1.77	*	0.45	0.87	ns
cis-pinocarvyl acetate	-	0.27	*	0.54	0.13	*
α-longipinene	0.74	0.28	ns	0.92	0.14	*
Nevyl acetate	0.68	0.69	ns	0.68	0.54	ns
Italicene	0.66	0.90	*	0.5	0.30	ns
E-Caryophyllene	40.49	27.05	*	48.21	40.13	ns
α-Humulene	8.87	3.34	*	10.38	2.91	*
E-β-Farnesene	2.79	5.77	*	3.21	14.99	*
7-epi-1,2-dehydro sesquicineol	0.68	0.76	ns	0.72	0.57	ns
γ-Muurolene	1.49	0.24	*	1.30	0.95	ns
Epi- cubenol	0.45	-	*	0.39	-	*
α-selinene	1.14	-	*	0.88	-	*
Spathulenol	9.36	33.87	*	6.83	41.54	*
Caryophyllen oxide	0.47	3.94	*	0.41	1.59	*
globulol	0.91	2.20	*	0.80	2.29	*
β-eudesmol	0.55	-	*	0.73	-	*
α-bisabolol	4.36	-	*	4.25	-	*

*P < 0.01, ns: not significant, F: flowering, S: seeding

Optimization of hydrodistillation and steam distillation methods for the other plant species has been previously studied. *Table 4* exhibits the summary of the number and concentrations of the components present in the volatile fractions, obtained by the HD and SD. There are evident differences between the percentage and the number of components, as follows: HD: 99.98% and 30, and SD: 94.87% and 23. The yield of volatile components of *P. ferulacea* obtained by use of the HD (0.29%) was higher than the SD (0.17%). This result is consistent with the findings of Kiran et al. (2005) and Sefidkon *et al.* (2007). The main components of these extractions were (E)-caryophyllene (48.21-52.26%), α-humulene (3.97-10.28%), spathulenol (6.73-10.37%), linalool (0.51-3.18%), and δ-3-carene (1.31-3.37%), among which the amounts of α-humulene and spathulenol were higher in the SD method than the HD method.

***Table 4**. Comparative chemical composition of P. ferulacea oil at flwering stage of plant growth by HD and SD methods*

Compound	**Flowering stage (%)**	
	hydro-distillation(HD)	Steam distillation (SD)
α-Thujene	0.84	-
Sabinene	0.72	0.49
Myrcene	1.19	0.69
p-Cymene	2.39	-
Limonene	2.18	0.51
δ-3-Carene	3.37	1.31

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γ-terpinene	-	0.49
Terpinolene	0.61	0.86
Dehydro linalool	1.29	1.25
Linalool	3.46	0.56
Octen-3-yl-acetate	0.83	-
Myrcenol	1.16	-
3-Octanol acetate	1.01	-
-1-Terpinoel	0.33	-
p-Cymene-8-ol	0.45	-
cis-pinocarvyl acetate	0.34	-
α-longipinene	0.82	-
Nevyl acetate	0.68	0.91
Italicene	0.5	0.70
E-Caryophyllene	48.21	52.26
α-Humulene	10.28	3.97
E-β-Farnesene	3.21	5.01
7-epi-1,2-dehydro sesquicineol	0.72	1.05
γ-Muurolene	1.30	1.54
Epi- cubenol	0.39	0.75
α-selinene	0.78	0.49
Spathulenol	6.73	10.37
Caryophyllen oxide	0.41	0.61
globulol	0.80	0.52
β-eudesmol	0.73	3.46
α-bisabolol	4.25	5.26
γ-himachalene	-	1.81
Total	99.98	94.87
Oil yield (% w/w)	0.29	0.17

Conclusions

According to the performed study, it can be concluded that based on the certain analyses of phenology and physiology, extending the extraction method can definitely increase the quality and quantity of the extracted essential oil of *P. ferulacea*.

On the basis of the findings, it can be suggested that the infertile individual at the flowering stage is a good sample for a comprehensive analysis of volatile compounds in *P. ferulacea* and can strongly enhance the quality of commercial oils. Moreover, while the SD and HD methods are relatively proper techniques for the quantitative analysis of volatile components, but it should be declared that extensive works are still required in order to adopt them for qualitative analysis.

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THREE SIDES OF THE SAME COIN? THE MAIN DIRECTIONS OF THE ENVIRONMENTAL MOVEMENT

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Abstract. Earlier publications about the main directions of the environmental movement have been incomplete. Some papers discussed the relationships between nature conservation and animal advocacy, but they either disregarded environmentalism, or treated it as a synonym of nature conservation. This latter is a conceptual error since it does not take into account the different primary objectives, distinct historical roots and dissimilar ethical standards of nature conservation and environmentalism. Although a few publications did recognise the difference between nature conservation and environmentalism, these papers failed to include animal advocacy into their analyses. Consequently, a comprehensive overview of all three directions of the environmental movement is still lacking. In this article we argue that the environmental movement has three main directions: (1) nature conservation, (2) environmentalism and (3) animal advocacy. We analyse their main objectives, historical roots and ethical standards, and we scrutinise their relationships to one another. Distinguishing the three main directions will ease the use of clear ethical arguments supporting specific decisions. By clarifying the relationships among the main branches, our conceptual scheme will help to find allies and solve conflicts in applied conservation, animal advocacy and environmentalism. If the three directions join forces, there will be some reason for optimism.
Keywords: *nature conservation, environmentalism, animal advocacy, environmental history, environmental ethics*

Introduction

It is a commonplace that, in an environmental sense, we are at a critical point in history (e.g. Vitousek et al., 1997; Williams and Crutzen, 2013). Given the various types of destructions we face and the enormous diversity of people who want to be part of the solution, it is no wonder that considerable differences exist among the different groups of the environmental movement. Differences range from minor variances in emphasis to desperate combats. A total harmony may be unrealistic and undesirable, but

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a clear and accurate definition of the main directions may contribute to the identification of powerful ethical arguments, reveal the causes of conflicts and help solve them, and result in a better co-operation.

As public environmental awareness has been rapidly increasing since the middle of the twentieth century, the environmental movement has also become more and more diverse. At the same time, it has become increasingly obvious that the environmental movement is not a solid block, but it consists of considerably different branches. However, there have been relatively few attempts to analyse the main directions and their characteristics, let alone their relationships.

As disagreements between conservation biologists and animal advocates came to light (with a debate occasionally resulting in strong exaggerations and misinterpretations on both sides; e.g. Callicott, 1980; Regan, 1983; Sagoff, 1984), considerable attention was paid to define these two directions. (We see that there is a fundamental difference within animal advocacy, namely between animal welfare and animal rights, but for the purposes of the present article, there is no need to treat them separately.) As a result, scientists and philosophers have regularly restricted their attention to only two branches of the environmental movement: animal advocacy and nature conservation (e.g. Callicott, 1988; Ehrenfeld, 1991; Midgley, 1992; Jamieson, 1998; Perry and Perry, 2008; Paquet and Darimont, 2010; Keulartz, 2015). The first problem with the above distinction is that it does not give the full picture: environmentalism is either totally disregarded, or it is merged with nature conservation. The lack of distinction between nature conservation and environmentalism is a general phenomenon (e.g. Western, 1989; Norton, 2000; Switzer, 2003; DesJardins, 2006; McShane, 2007; O'Neill et al., 2008), the cause of which probably lies in the fact that the words 'nature' and 'environment' are used as synonyms. Fusing nature conservation and environmentalism is a conceptual error, which does not take into account their different primary objectives, distinct historical roots and dissimilar ethical standards. The second problem is that the conclusions concerning the relationships between conservation and animal advocacy were highly variable, from total irreconcilability (e.g. Saggoff, 1984) (*Fig. 1a*) to compatibility (e.g. Jamieson, 1998) (*Fig. 1b*).

There are extremely few publications that distinguish between nature conservation and environmentalism, recognising their different primary purposes (Margóczi, 1998; Heiland, 1999; Foreman, 2006; Gallé, 2013), or the different purposes plus the distinct historical traditions (Noss, 1999; Hunter and Gibbs, 2007). Unfortunately, the relationships between the two directions have never been explored in detail, although some publications noted that they are overlapping (e.g. Margóczi, 1998). In addition, none of the above publications regard animal advocacy as belonging to the environmental movement (*Fig. 1c*), which is unfortunate, to say the least.

Although Foreman (1991) clearly differentiated among the three main directions (i.e. nature conservation, environmentalism, animal advocacy), this was only in a brief footnote, without further considerations or analyses. The idea has not gotten much scientific attention nor has it been elucidated in detail.

In sum, a careful examination of the main aims, ethical principles and historical roots of the three directions within the environmental movement is still lacking. Also, their complex relationships have never been examined thoroughly, which may have far-reaching theoretical and practical consequences.

In this article we argue that it is convenient and useful to distinguish between nature conservation and environmentalism, for several reasons. First, environmentalism is

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usually concerned about human welfare and human environment, which does not apply to conservation (Noss, 1999; Foreman, 2006; Hunter and Gibbs, 2007). Second, as a consequence of the above distinction, the ethical foundations differ considerably, environmentalism having a more human-centered focus, while conservation being non-anthropocentric (Margóczi, 1998). Third, the two branches have a rather different history: nature conservation has its roots in the nineteenth century activity of distinguished American writers and naturalists such as Ralph Waldo Emerson, Henry David Thoreau and John Muir, whereas environmentalism emerged in the second half of the twentieth century, marked by notable figures such as Rachel Carson, Barry Commoner and Denis Hayes (cf. Strong, 1988; Switzer, 2003; Hunter and Gibbs, 2007).

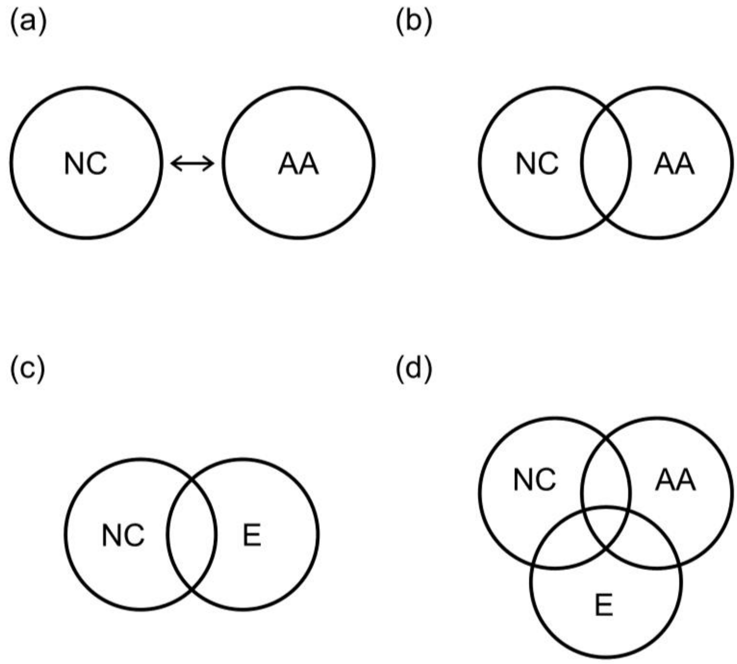

Figure 1. *Relationships among the main directions of the environmental movement. (a) Concepts exploring nature conservation and animal advocacy, regarding them as irreconcilable, (b) Concepts exploring nature conservation and animal advocacy, realising their compatible and overlapping character, (c) Concepts exploring nature conservation and environmentalism, regarding them as overlapping, (d) Our conceptual scheme exploring all three directions with considerable overlaps. NC: nature conservation, AA: animal advocacy (animal rights/welfare activism), E: environmentalism.*

The conceptual scheme to be described in the present paper was built on the embryonic outlines of the three-way solution of Foreman (1991). Thus, unlike the overwhelming majority of the earlier publications, we suggest that three main directions should be recognised within the environmental movement: (1) nature conservation, (2) environmentalism, and (3) animal advocacy. We make an attempt to identify the primary aims of the three branches. We also give a basic insight into how different their historical backgrounds are. In addition, we try to identify the ethical principles underlying the three main directions. Our further goal is to clarify their relationships to one another. In doing so, we give some examples how a better co-operation of the three directions may be possible.

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We think that our clarification and the resulting conceptual scheme have the potential to contribute to a better usage of well-founded ethical arguments in applied nature conservation, environmentalism, and animal advocacy. Furthermore, areas of overlaps (where the aims of two or three directions converge) can be identified, which can help to find allies for particular environmental measures or actions. In other cases, our scheme may reveal the reasons underlying specific conflicts, and may help solve them. We hope our present work will contribute to an increased efficiency of the environmental movement.

The main directions and their historical roots

Nature conservation is often defined as an activity that aims to conserve species, natural communities, ecosystems and ecological processes (e.g. Soulé and Wilcox, 1980; Ehrlich and Ehrlich, 1981; Soulé, 1985, 1986; Primack, 1993, 2004; Begon et al., 1996; Gaston, 1998; Mascia et al., 2003; Borgerhoff Mulder and Coppolillo, 2005; Meffe et al., 2006). The primary focus of nature conservation is biodiversity; it intends to prevent or minimise unwanted loss in the diversity of life at several levels (e.g. genetic diversity of populations, species diversity, habitat diversity). It is important to emphasise that nature conservation does not necessarily intend to reach the highest possible biodiversity (it would be nonsense to plant invasive species into a species-poor habitat to increase its diversity). Instead, it seeks to maintain an appropriate level of diversity. In sum, nature conservation focuses on species, communities, and it does not engage in the welfare of individuals (Soulé, 1985).

The focus of nature conservation on natural and near-natural habitats, processes and diversity is understandable if one considers the beginnings of modern conservation. Till the nineteenth century, at least in the Western world, nature (or wilderness) was considered worthless, hostile and undesirable, something that had to be destroyed, tamed and exploited (Cronon, 1996; Herrmann, 2007). Among the first ones who recognised the aesthetic and spiritual values of nature was Emerson (1836). His followers, most notably Thoreau (1854) and Muir (1912) were fascinated by the wilderness, and took every opportunity to observe its beauties. For them, wilderness areas were sacred places. Their influence culminated in the work of Leopold (1949), who is rightly considered the father of nature conservation.

As for environmentalism, first we have to determine whose environment we want to protect. It has been recognised that there is no such thing as a common environment, which would be the same for every living being. Instead, there are at least as many environments as there are organisms (Juhász-Nagy, 1984, 1986; Bartholomew, 1987; Heiland, 1999; O'Neill et al., 2008). It seems clear that the primary aim of environmentalism is to optimise the environmental parameters of the human species (Margóczi, 1998; Heiland, 1999; Noss, 1999). The primary interest of environmentalism is not wilderness, nor ecosystems, nor diversity, but human environment. Preventing smog formation, reducing soil contamination, providing safe drinking water, protecting the ozone layer, lessening soil erosion, and so on, are all human-centered actions (although they are clearly beneficial for a variety of other organisms as well). Even combatting global climate change, one of today's main focal points in environmentalism, is mainly interested in the future of the human species, although we do not deny that concerns do exist for other species and natural communities as well.

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Environmentalism is considerably younger than nature conservation. The start of modern environmentalism is marked by the now legendary book of Carson (1962). Even though the horrible effects of pesticides on birds and other living creatures were a central issue, considerable attention was paid to the negative influences on human health and human environment. Other classics during this initial period of environmentalism also focused primarily on energy issues, air pollution, soil contamination, human overpopulation and climate change, while biodiversity was discussed as a marginal topic, or was not discussed at all (e.g. Boulding, 1966; Hardin, 1968; Meadows et al., 1972; Schumacher, 1973; Hayes, 1977; Gore, 1992). Thus, the focus clearly differs from that of the nineteenth century conservation (see also Wildes, 1995).

Animal advocacy is interested in the rights and/or well-being of individual animals. It does not focus on species but on individual organisms who have interests, welfare or a quality of life (Ehrenfeld, 1991). It aims to cease or lessen animal mortality and suffering evoked by humans.

Of the three directions discussed in this paper, animal advocacy has the longest tradition, dating back to at least the eighteenth century, with one of the most prominent forerunners being utilitarian philosopher Jeremy Bentham (Guither, 1998; Beers, 2006). In the nineteenth century the movement spread primarily in the UK and the US (Guither, 1998), and it gained new momentum in the twentieth century, with the publication of three landmark books in animal advocacy (Ryder, 1975; Singer, 1975; Regan, 1983).

Ethical foundations

If the three main directions of the environmental movement are recognised, the corresponding ethical bases should also be identified.

As noted by Soulé (1985), conservation biology tends to be holistic. It seems clear that so does conservation activity. Thus, nature conservation focuses on biological levels above individuals. In an ideal case, nature conservation is not human-centered (Soulé, 1985; Barry and Oelschlaeger, 1996). Correspondingly, the underlying ethical principle must be holistic and non-anthropocentric. The statement of Leopold (1949) may serve as a useful guiding principle: ‘A thing is right when it tends to preserve the integrity, stability, and beauty of the biotic community. It is wrong when it tends otherwise.’ This is an ecocentric ethic, as it focuses neither on humans nor on the welfare of individual organisms, but on whole ecological systems. Although other targets of nature conservation (species, processes, etc.) are not mentioned in this short normative claim, it is obvious that these are necessary for the integrity, stability and beauty of a community. Similar arguments emphasizing the non-instrumental (intrinsic) value of nature had appeared earlier (e.g. Muir, 1912), but Leopold’s (1949) effect on current conservation ethics proved to be the greatest. In fact, according to Callicott (1990), a correct interpretation of Leopold’s (1949) famous maxim is able to guide our current conservation activities.

One may argue that nature conservation is, to some extent, anthropocentric. In fact, some researchers differentiate between ‘resource conservation’ and ‘preservation’ (e.g. Callicott, 1990; Hunter and Gibbs, 2007). The distinction is rooted in the nineteenth century history of the movement, when ‘resource conservation’ placed an emphasis on an optimal use of natural resources for the benefit of present and future human

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generations, while ‘preservation’ favoured setting aside large natural areas where any major human activity should be prevented (Wildes, 1995; Wellock, 2007). Nowadays, both subfields are placed under the umbrella of conservation (Noss, 1999), but it is easy to recognise the human-centered view of the first approach. However, as pointed out by Madhusudan and Shankar Raman (2003) and Kareiva (2014), the two concepts are additive rather than mutually exclusive. Even though some conservation stakeholders place more emphasis on anthropocentric reasons for protecting nature, research has shown that most if not all of them share some fundamental ecocentric values (Berry et al., 2016). Scientists and practitioners have recognised that to gain public support for conservation efforts, we have to emphasise nature’s goods and services for humans (e.g. Norton, 1991; Odenbaugh, 2003; see also Reyers et al., 2010; Lele et al., 2013). Nevertheless, the primary and most important reason to protect nature, at least for most conservationists, is nature itself (cf. Ehrenfeld, 1976; Ehrlich and Ehrlich, 1981; Soulé, 1985; Barry and Oelschlaeger, 1996; Foreman, 2006; McShane, 2007). Even conservationists who usually emphasise instrumental values may well prove ecocentric. Consider, for example, the statement of Kareiva (2014): ‘I would prefer a world in which everyone believes that saving nature for nature’s sake is simply the right thing to do and a moral imperative.’ This is clearly an ecocentric view, even though he is regarded as rather anthropocentric (Hunter et al., 2014). The recognition of the benefits of nature for humans does not annul conservationists’ thinking about the non-instrumental value of nature (Ehrenfeld, 1976). In addition, anthropocentric reasons for conservation efforts may have serious limits, while ecocentrism may be a more robust ethical foundation for nature conservation (Ehrenfeld, 1976; Callicott, 2002; Deliège and Neuteleers, 2015). Strongly related to this topic is the debate concerning the ecosystem services approach, especially the economic valuation of ecosystem services (e.g. Ridder, 2008; Norgaard, 2010; Gómez-Baggethun and Ruiz-Pérez, 2011; Salles, 2011; Costanza et al., 2014). In sum, we firmly believe that, at heart, nature conservation should be ecocentric. In contrast, disciplines and management activities with an exclusively anthropocentric base, focusing solely on the sustainable human use of resources (such as wildlife management or forestry) may be termed resource management or resourcism, but they should not be confused with nature conservation (Noss, 1999; Foreman, 2006).

As discussed above, environmentalism is primarily focused on humans. Therefore, it is reasonable to think that the underlying ethical principle is human-centered, i.e. anthropocentric. However, it is important to emphasise that this anthropocentrism is not equal to the conventionally accepted meaning of anthropocentrism. Anthropocentrism usually states that only humans have intrinsic value (or at least they have the largest intrinsic value), or that only humans are morally considerable (cf. Callicott, 2006; DesJardins, 2006; Nolt, 2015). We think that most environmentalists would strongly object to such a statement. Instead, environmentalism focuses on one species, ours, by protecting the environment we live in, without claiming that other organisms or ecological entities are less valuable. This concept may be termed ‘environmental anthropocentrism’. Moreover, it is also clear that numerous other species also benefit from protecting the human environment.

At the base of animal advocacy (including both animal rights and animal welfare activism), there are individualistic ethics. Among individualistic ethics, sentiocentrism and biocentrism have to be evaluated. Both the utilitarian arguments of Singer (1975) and the rights-based theory of Regan (1983) pay major attention to the ability of feeling

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pain and pleasure. Therefore, both views can be regarded as sentiocentric (Callicott, 2006) or pathocentric (Wolf, 1996; Krebs, 1997). Biocentrism appears if our moral horizon is further expanded to include all living beings, as was done, for instance, by Schweitzer (1923), Goodpaster (1978) and Taylor (1981). Both sentiocentric and biocentric ethics are potential candidates for the ethical base of animal advocacy, since both concentrate on individuals. Nevertheless, sentiocentrism, focusing on sentient animals (and disregarding individual plants), seems to be more closely connected to current animal advocacy issues. It has to be noted that since Darwin (1859, 1871), sentiocentrism has a firm scientific basis, while traditional anthropocentric views are undermined and out-dated (see also Rachels, 1990; Erdős, 2015; Puryear et al., 2017).

It is doubtful whether one single ethical theory is capable of guiding our decisions in all of the difficult environmental situations (Norton, 2000). Instead, a value pluralism may be a better choice (Norton, 2000; Minteer and Collins, 2005; DesJardins, 2006; Carter, 2011; also see Wenz, 1993). Minteer and Collins (2005) argued that in many cases, nature-centered (i.e. ecocentric), human-centered (i.e. anthropocentric) and individual-centered (i.e. sentiocentric or biocentric) arguments should be considered simultaneously. For example, during ecological studies or conservation actions focusing on habitats or ecosystems, not only the holistic viewpoint (ecocentrism), but also animal welfare issues (sentiocentrism) and the effects on humans (anthropocentrism) should be taken into account.

Thus none of the above three ethical bases suggested for nature conservation, animal advocacy and environmentalism (ecocentrism, sentiocentrism and environmental anthropocentrism, respectively) should be considered universally valid. A careful examination and balance of these principles is needed, especially in the most difficult and complex cases.

Areas of conflict and co-operation

Although the three main directions of the environmental movement are separable, in the followings, we will demonstrate that they overlap considerably (*Fig. 1d*). If, for example, a tropical rainforest is set aside as a reserve, this may be considered an action that belongs to nature conservation, since the primary aim was probably the preservation of habitats and species. At the same time, however, large amounts of carbon are sequestrated in the biomass, which would contribute to global warming if the forest was destroyed. Thus, the designation of the reserve fits the goals of environmentalism. Finally, it also fits animal advocacy viewpoints, for it is quite obvious that no wild animal has a chance to a decent life without a natural habitat (cf. Taylor, 1981; Jamieson, 1998; Paquet and Darimont, 2010).

We do not deny that conflicts arise, but the above example shows that a co-operation of the three branches is possible and desirable. To give an exhaustive analysis of conflicts and solutions is not the aim of the present paper. In the following sections, we only want to give a basic insight into areas where conflicts may emerge, and fields where there are great potentials for co-operation. We will focus on the relationship between nature conservation and animal advocacy, but the other two pairs of the triangle will also be discussed briefly.

Nature conservation versus animal advocacy

Confrontation, as already noted in the Introduction, is well-known between nature conservationists and animal advocates. The cause is simple: animal advocates care for individuals, while nature conservation is interested in species, habitats, etc. However, the common points should not be neglected either. For example, both branches are put under the umbrella of 'green issues' by the public (Perry and Perry, 2008). Most conservationists do care for individual's welfare, and most animal advocates value natural communities (Perry and Perry, 2008). In addition, the membership of animal advocacy and conservation groups overlaps considerably (Ehrenfeld, 1991; Jamieson, 1998). Finally, they form a common platform, since both directions reject anthropocentric views common and dominant in western societies (Callicott, 1988; Paquet and Darimont, 2010). It has been shown that open and rational dialogues between the two groups may reveal that, despite some differences, they do agree concerning several issues (Thompson and Lapointe, 1995). As Aitken (1997) noted, concerns for individual animals do not necessarily contradict conservation goals.

Conservation of a habitat or a species very often requires the elimination of a non-native animal species, and the killing of the unwanted individuals may seem a plausible solution, which, quite naturally, is not welcome by animal advocates. However, even in this seemingly inextricable case, the co-operation has proven possible and mutually beneficial (Perry and Perry, 2008). First of all, preventing the establishment of potential invasive species corresponds to the ethical principles of both directions. Second, if the invasive species has already arrived, alternative measures such as live-trapping and subsequent neutering or translocation (instead of killing) should be discussed between animal advocates and conservationists as early as possible. Perry and Perry (2008) concluded that the two groups should make every effort to find a common ground, and this could result in conservation actions that are acceptable for both parties. Killing invasive species all too often occurs routinely and easily, and killing is usually carried out in the name of conservation even if it serves other goals (van Dooren, 2011). We think conservationists and animal advocates agree that similar practices should be abandoned.

Feral and unowned cats may cause considerable damage to native fauna (Longcore et al., 2009) (although some argue that they only have a limited and short-term effect on native bird and mammal populations; Jarvis, 1990). As a possible solution to reduce the negative impacts on wildlife, the killing of the cats is usually proposed, which is unacceptable for most animal advocates. As a compromise, trap-neuter-release (TNR) programs have been introduced as non-lethal alternatives, during which cats are sterilised and put back where they were trapped. Although animal advocates claim that the method is efficient (e.g. Levy et al., 2003), conservationists have serious doubts and state that cat colony sizes do not decrease fast enough (e.g. Longcore et al., 2009). Irreconcilable as the debate may seem, the common ground is easily reached when we consider that the long-term reduction in the number of stray animals is the goal for both sides. For this, neither lethal methods, nor TNR-programs will be satisfactory; rather, the source of the problem has to be treated. The propagation of responsible animal keeping is necessary, to prevent steady new supplies to free-roaming cat populations. Encouraging citizens to neuter their cats would contribute to a significant decrease in unwanted reproduction, lessening the damage to natural values and at the same time avoiding unnecessary animal suffering (Jarvis, 1990). For a fruitful co-operation, animal advocates have to accept the concern of conservationists for wildlife, and

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conservationists should appreciate cat-lovers' enormous efforts in neutering and adopting unowned cats, often without any funding. It should also be kept in mind that TNR-schemes combined with adopting of at least some of the cats can bring relatively fast successes in the reduction of unowned cat population sizes (Levy et al., 2003).

One of the most promising areas of co-operation between nature conservation and animal advocacy groups is, somewhat surprisingly, meat production. Tens of billions of animals are confined to extremely small areas in 'factory farms' worldwide, living under terrible conditions (Nierenberg, 2006; Halweil and Nierenberg, 2008). At the same time, at least in many European and Asian temperate landscapes, grazing is considered an efficient conservation management, yet several valuable grasslands are undergrazed or not grazed at all (Zahn et al., 2007; Peeters, 2009; Erdős et al., 2011; Kiss et al., 2011; Házi et al., 2012; Saláta et al., 2012; Wichmann et al., 2013; Mardari and Tănase, 2016). Market trends clearly show that consumers prefer animal products that have been produced according to stronger animal welfare standards (i.e. products that originate from free-ranging animals) (e.g. Phan-Huy and Fawaz, 2003; Halweil and Nierenberg, 2008; Ventura et al., 2015). It is not difficult to recognise the common ground of conservationists and animal welfarists, although it has to be noted that those who hold the animal rights position would not join this platform, since they refuse any kind of animal exploitation, irrespective of how the animals are kept (e.g. Regan, 1983; Francione, 2008). However, even they admit that free-ranging animals have a better life than those living in confinement.

Another promising area of co-operation is the action against the overharvest of animal populations, which is a serious conservation threat, and at the same time it has obvious effects on the animals' welfare. Whaling, for example, is in the crosshairs of both animal advocates and conservationists. In addition, the (mainly illegal) trade of other animals and animal products should also be considered in this regard. Baker et al. (2013) suggested that an efficient collaboration between animal advocates and conservationists would be most welcome in issues like this.

Not only do some animal welfare issues have a practical conservation relevance, but they may be of scientific importance as well. For example, toe clipping of amphibians in mark-recapture surveys (the removing of a combination of their digits) has been a wide-spread tool. Animal advocates' arguments against the unethical practice may have been automatically rejected and declared 'unscientific' once, but as we now know, the method in fact distorts scientific results and has conservation effects due to reduced survival of marked individuals (May, 2004).

Nature conservation versus environmentalism

A conflict has recently come to light between the use of renewable energy and the conservation of natural values, as certain disadvantages of alternative energy sources have been revealed. For example, the large-scale application of solar energy facilities in natural habitats (e.g. deserts) has various direct and indirect detrimental influences on native species (Lovich and Ennen, 2011). Wind turbines also proved to have serious adverse effects on wildlife (Kuvlesky et al., 2007; Lin 2017). Although negative impacts may not be completely eliminated, they can be minimised through careful planning (e.g. Stewart et al., 2007; Lin 2017). As the need for renewable energy increases, the co-operation of environmental engineers and conservation biologists is desperately needed.

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Strongly related to this issue are the adverse effects of biomass production for providing 'green' energy, which usually needs huge areas of land. Needless to say, biomass monocultures of fast-growing plants totally disrupt native communities (e.g. Abbasi and Abbasi, 2010; Gomiero et al., 2010) and are often objected by conservationists. However, the conflict is not as serious as it may seem at first glance. For example, biomass production that results in deforestation is not only unwanted from a conservation perspective, but it is questionable from an environmentalist's point of view, since its net effect is likely to exacerbate global warming (Field et al., 2007). A compromise may be possible in some cases, if, for example, traditional agroforestry practices using biomass energy are re-established (Plieninger and Bens, 2008), or used cooking oil is converted to biofuel.

Animal advocacy versus environmentalism

Conflicts between animal advocate groups and environmentalists have probably not been as apparent as in the previous two cases. Nonetheless, it should be mentioned that environmentalists sometimes refer to the fact that keeping companion animals is environmentally destructive, mainly because of the meat-based diet of the pets (Rushforth and Moreau, 2013). However, negative impacts can be reduced with conscious selection of the pet food type. Moreover, it should be kept in mind that one of the main goals of the animal advocacy groups is the reduction in the number of companion animals, through the propagation of neutering and responsible animal keeping. Moreover, some animal advocates strongly oppose the breeding of pets, claiming that no more animals should be brought into existence just to be abused or exploited later by cruel or indifferent humans (Francione, 2008). (Of course, animal advocates insist that we must care for animals who are already alive.) In fact, what seems to be a conflict can turn into a common ground by close inspection: Action against careless or profit-oriented breeding reduces environmental harm and avoids unnecessary animal suffering.

It is well-known that, besides being responsible for incredible animal suffering, intensive animal farms also have highly destructive effects on the environment by producing greenhouse gases and causing water pollution (Koneswaran and Nierenberg, 2008; Ilea, 2009; Rossi and Garner, 2014; Waldau, 2011). Several solutions may be possible, ranging from a reduction in meat consumption (e.g. Halweil and Nierenberg, 2008) to the avoidance of products from 'factory farms' (e.g. Appleby, 2005) to becoming vegetarian or vegan (e.g. Hill, 1996), all of which benefit animals and the environment at the same time (e.g. Goodland, 1997; Jamieson, 1998; Halweil and Nierenberg, 2008; Jankielsohn, 2015). In some or most of the above cases, there is much room for animal advocates and environmentalists to co-operate.

Conclusions

To efficiently combat today's various environmental challenges, we have to see where we have come from, and what our ultimate goal is. In this paper, expanding the ideas of Margóczi (1998), Noss (1999) and Hunter and Gibbs (2007), we differentiated between environmentalism and nature conservation. As a result, building on the proposal of Foreman (1991), we identified and defined the three main directions of the environmental movement: nature conservation, environmentalism and animal advocacy. We are aware that the three areas overlap considerably. We also

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see that they may further be subdivided. Nevertheless, we think that the use of the three basic categories is justified by their different emphases, distinct ethical principles and different historical roots.

Although conflicts do exist among the three directions, we think that they can be overcome if we understand the underlying reasons. Members of the three directions should try to understand each other's views. As we demonstrated with some examples, the areas for co-operation are huge and promising (*Fig. 1d*). Not only are the three directions reconcilable in most cases, they have much in common.

Earlier works either focused on nature conservation and animal advocacy, and disregarded environmentalism, or examined nature conservation and environmentalism, but did not consider animal advocacy. As a result, the complex inter-relationships among the three directions remained unexplored. In contrast, our conceptual scheme takes into account all three directions. One advantage of this is that it may help reveal ethical or historical reasons behind specific conflicts. On the other hand, it may ease the identification of convergences, which could support finding allies for particular measures or actions. All directions of the environmental movement typically have serious budgetary, staff and time constraints. Thus, both the rapid solution of conflicts and joining forces in converging situations may be essential if we are to increase the effectiveness of the environmental movement. We hope our conceptual scheme is one step in that direction.

As it was suggested in the previous sections, challenging humankind's present meat production and consumption practices is a focal point where overlaps exist among the different directions. First, it seems clear that a shift from an animal-based towards a plant-based diet in Western countries could be a win-win-win situation for nature conservation, environmentalism and animal advocacy. Nature conservationists may consider that 'the livestock sector may well be the leading player in the reduction of biodiversity' (Steinfeld et al., 2006). Environmentalists can be concerned about livestock's resource intensity (requiring a lot of agricultural land, water and energy) and its high levels of pollution (emissions of greenhouse gases, acidifying gases, reactive nitrogen compounds, veterinary medicines and pesticides). Animal advocates care about the immense suffering of farm animals. Thus, cutting down meat consumption could be one of the great challenges of the twenty-first century (Rösch, 2002, Stoll-Kleemann, 2014). It must be emphasized that, as high meat consumption has negative social and health effects, producing less meat could mean a win situation for other fields beyond the environmental movement as well (e.g. Hill, 1996; Stoll-Kleemann, 2014, Westhoek et al., 2014).

Second, animal-friendly and environmentally friendly methods should be supported. According to Appleby (2005), improved animal welfare in farms promotes environmental sustainability and vice versa, because both approaches regard agriculture mainly as a biological rather than a mere technological process. At the very least, meat and other animal products from cropland and resource intensive low-nature-value farming systems, including 'factory farms', should be avoided. Unfortunately, even small-scale farms are environmentally harmful and animals have in some cases as miserable lives as in intensive farming. It is clear that we should make every effort to avoid animal products from each of these deeply unethical sources. It is important to point out here that, according to Curry (2011), organic and animal-friendly farming would be able to feed the whole human population on Earth, provided that global meat consumption is limited.

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Third, adopting a vegetarian or vegan lifestyle will have the greatest positive effect on animals, nature, and the environment. Pluhar (2009) concluded that a vegetarian diet (and possibly in-vitro meat production) may be the best alternative to factory farming, while Deckers (2009) claims that veganism is even better and more consistent both from an environmentalist's and an animal advocate's point of view.

The above examples clearly show that challenging intensive and cruel animal farms is a key point where animal advocates, conservationists, and environmentalists can agree and work together (Regan, 1983; DeGrazia, 2002; Halweil and Nierenberg, 2008; Gjerris et al., 2011; Gjerris, 2015; Hayes and Hayes, 2015). However, it sometimes appears that a strong collaboration between the nature conservation, environmental and animal advocacy movements is hindered by an obstacle, a psychological bias. When there is too much justification, such as in a win-win situation, motivation can be undermined. Regarding a shift in diet away from animal products, some environmentalists seem scared to talk about animal suffering. Meanwhile, some animal rights activists invest a lot of time and energy criticising other people who eat vegan or want to become vegan due to health or environmental reasons. Animal advocates think that the health and environmental vegans have the wrong objectives and some even believe that advocating veganism for health and environmental reasons is counterproductive. They believe that one should always and only refer to the animal rights objective when promoting veganism. This phenomenon is also at work in other areas, such as family planning (access to contraceptives to prevent unwanted pregnancies): women rights activists who campaign for family planning as a reproductive right often criticize environmentalists who propose family planning as an effective means to limit the environmental impact from human overpopulation. According to those feminist advocates, environmental concerns should not be a valid objective for promoting family planning. Reversely, many environmentalists and conservationists are reluctant to talk about family planning as a means to decrease population growth. We can call this pyschological phenomenon the 'single objective bias', which can be strong enough to generate mutual hostility between the movements. When this single objective bias can be overcome, all three movements will gain strong benefits in an effective collaboration.

We are facing great challenges, and there is no doubt that solutions will be easier with co-operation. Probably the best-known example for synergism among the main directions of the environmental movement is provided by Jane Goodall, who is a dedicated conservationist, an animal advocate and an environmentalist at the same time (see for example: Goodall and Berman, 1999; Goodall and Bekoff, 2002; Goodall et al., 2005).

Besides being a role-model for millions, Jane Goodall has also been a lifelong optimist. If conservationists, environmentalists and animal advocates join forces, perhaps there will be some reason for optimism.

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EFFECTS OF THE SURFACE CRACKS CAUSED BY COAL MINING ON SOIL CHARACTERISTICS AND WHEAT GROWTH IN HUANG-HUAI-HAI PLAIN, CHINA

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Abstract. Coal has provided a great proportion of energy supply for the country. However, coal mining has caused lots of ecological and environmental problems. In this study, a field investigation experiment was conducted in subsided area of Zhaogu coal mine of Jiaozuo Coal Group, Henan Province, China, to explore effect of surface cracks caused by coal mining on the soil characteristics and crop growth. The results showed that during the jointing stage of wheat, the ranges within which the larger crack (C-1) and smaller crack (C-2) caused the effects on both soil water content and soil respiration were 0-60 cm and 0-30 cm from the cracks, respectively. C-1 significantly reduced the activities of soil urease and invertase within 0-60 cm from the crack but C-2 did not cause significant inhibition on soil enzymes. During the flowing stage of wheat, C-1 and C-2 significantly reduced soil water content, available nitrogen content and soil respiration within 0-60 cm and 0-30 cm from the cracks, respectively. The range within which C-1 caused inhibition on urease activity was 0-60 cm while such range for C-2 was 0-30 cm from the crack. The ranges within which both C-1 and C-2 caused significant inhibition on invertase activity were 0-60 cm from the cracks. C-1 and C-2 caused significant inhibition on photosynthesis of wheat within 0-60 cm and 0-30cm from the cracks, respectively. C-1 and C2 significantly reduced spike number and grain yield within 0-90 and 0-60 cm from the cracks, respectively. In conclusion, surface cracks resulted in loss of water and nutrients and reduction in crop yield, which is more serious with lager cracks. Therefore, further studies are needed to address this problem and to find effective measures to resolve it.
Keywords: *coal mining subsidence; coal mine; characteristics of soil microorganisms; soil water; fertilizer*

Introduction

Coal is the major energy source in China. Coal mining industry in Henan province is ranked the second place in China and its coal output accounts for nearly 10% of the country's total (Hu et al., 2014a). While the coal mining areas in Henan have provided substantial proportion of energy supply for the country, some ecological and environmental problems have been caused by coal mining, especially underground coal mining, which have become increasingly serious. Underground coal mining leads to large-scale surface settlement, increases the original geomorphological slope and generates a large number of cracks (gaps), causing substantial damage to the ecological environment in the mining areas (Kuang and Deng, 2007; Tripathi et al., 2009). Under the comprehensive interactions of the subsidence disturbance, rain and wind, the loss of water and fertilizer and soil erosion can easily take place in the farmland in the subsided areas, leading to the degeneration of soil quality (Hu et al., 2014b). Furthermore, due to the uneven surface and the distribution of cracks/gaps all over the subsided areas, the

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soil evaporation area and evaporation intensity are not only increased, the farmlands also loss irrigation capacity. Their drought-resisting capabilities are greatly weakened, which, in turn, seriously affects the farmland productivity. Additionally, coal mining also destroys the structure of the water-holding layer of soil and causes a series of problems such as the declined phreatic table, drought well and the death of vegetation etc (Kuang and Deng, 2007). Therefore, to investigate the effects of cracks caused by coal mining on soil characteristics and crop production is of practical significance and theoretical value for reclamation and management of the farmland in coal mining areas.

In the recent years, a large number of studies have been conducted on the damaged farmland in coal mining areas. However, these studies mainly focused on such aspects as the effects of mining subsidence on physicochemical characteristics of soil (Li et al., 2010; Chen et al., 1999; Sadhu et al., 2012), the surface vegetation (Zhou et al., 2009; Quan et al., 2006; Moreno-de las Heras et al., 2008; Brom et al., 2013), quality of farmland (Meng et al., 2009; Martinez et al., 2013; Hossain et al., 2015) and restoration technologies of the damaged farmland (Cheng et al., 2014) etc. While a few studies have been conducted on the mechanisms underlying the formation of cracks/gaps (Wu et al., 2009) and the effects of cracks on soil water (Zou et al., 2013; Wei et al., 2006; Zhang et al., 2007), very few reports about the temporal and spatial changes in the characteristics of the soil surrounding the cracks/gaps and the effects of cracks on plant growth and development have been available. In the subsided areas, the newly formed micro-geomorphic features such as subsided hole, subsided cave, and cracks (gap) etc. caused by mining subsidence also bring about corresponding changes in the spatial structures of soil. The changes in the spatial factors that affect the soil characteristics certainly cause the spatial changes in the physicochemical properties of the surrounding soil (Zhao et al., 2010). The cracks (gaps) in the soil lead to the increase in the evaporation area and evaporation intensity of soil in subsided areas. The disturbance of cracks on soil water affects not only soil physicochemical properties but also the characteristics of soil microorganisms (Li et al., 2014) and ultimately affects the growth of plants. Thus, strengthening the studies in this aspect will be beneficial for complete understanding and evaluating the effects of coal mining on the regional environment.

In this study, by choosing the farmland located in the subsided area of Zhaogu Second Coal Mine of Jiaozuo Coal Group, Henan Province, China, as the research object, a field investigation and laboratory analysis experiment was conducted to explore effect of surface cracks caused by coal mining on the soil characteristics and crop growth in different locations surrounding the cracks, which may provide the theoretical basis for reclamation/management of farmland and for improvement of the productivity of farmland in coal mining subsidence area.

Materials and methods

Site description

In this experiment, the subsided area of the Zhaogu Second Coal Mine of Jiaozuo Coal Group, Henan province, China, was selected as the studied area (*Fig. 1*). The mean annual atmosphere temperature was 14^{o}C, the mean annual precipitation was 603-713 mm and the mean water evaporation intensity was 2039 mm. The air temperature and precipitation during the wheat growing season are shown in *Table 1* and *Figure 2*, respectively. During the recent years, the large-scale coal mining has caused serious damage to the local farmland. Approximately 40 hm^2 subsided area have been formed,

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among which, approximately 30 hm^2 was the stable subsided region and 10 hm^2 was the dynamically subsided region (Xu et al., 2015). There are many cracks (gaps) caused by surface subsidence in the dynamically subsided region, which led to the serious loss of soil water and nutrients. This seriously affected not only the quality of soil environment but also the regional agricultural production.

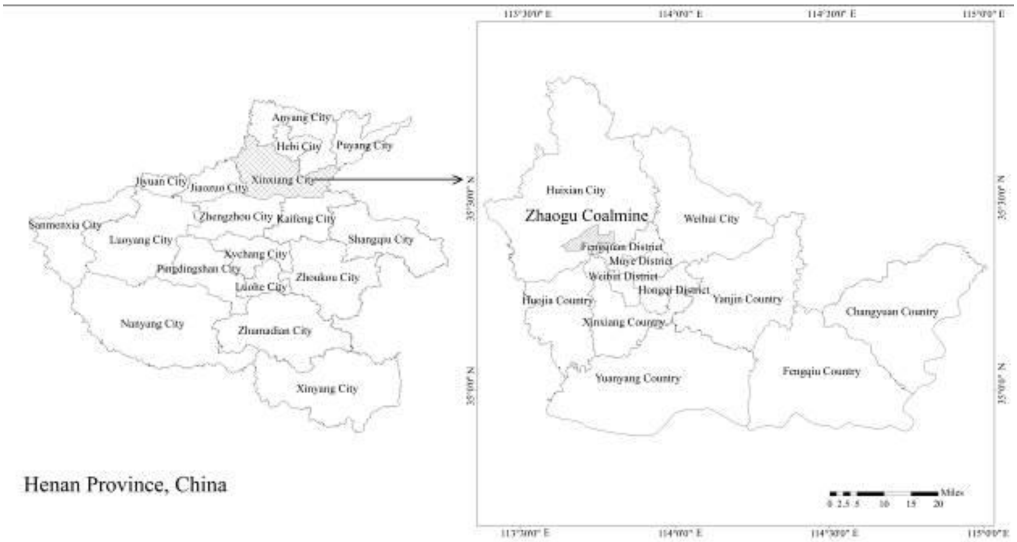

Figure 1. *The location of the sampling site.*

Figure 2. *Distribution of the monthly precipitation at the study area*

Table 1. *Distribution of the monthly air temperature at the study area*

Months	Jan.	Feb.	Mar.	Apr.	May	Jun.
High temperature	16.9	14.9	28.5	29.0	38.2	39.7
Low temperature	-5.5	-9.1	1.4	7.6	10.7	19.4
Average	2.9	2.6	12.7	17.1	29.7	32.0

Sampling methods

This experiment was conducted in the subsided farmland in Zhaogu Second Coal Mine from February to June, 2014. The soil was silty clay loam. For studying the effects of subsided cracks on the crop growth, two vertical, obviously well-developed cracks in this subsided area were selected as the studied objects. Among which, the

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length, width and depth of larger crack named Crack 1(C-1) were 45 m, 5-10 cm and 200 cm, respectively. The length, width and depth of smaller crack named Crack 2 (C-2) were approximately 25 m, 1-3 cm and 100 cm, respectively. These cracks had been formed for about 30 days. Soil and plants sample in the locations with 30, 60, 90 and 120 cm from the edges of the cracks were collected. The physicochemical properties of soil and physiological and ecological characteristics of wheat were examined. The soil and plant samples in the locations with different distances from the edge of cracks collected three times at each side of the cracks with the total of 6 times of collecting these samples.

The experimental crop was winter wheat (Bainong Aikang 58) with the mean planting density of 1.8×10^6 plants /hm^2. At both the jointing and the flowering stages, soil samples at 0-30 cm and 30-60 cm layers were collected were collected by using auger boring at each sampling point. These soil samples were immediately put into the aluminum box and sealed completely, and then brought back to laboratory for subsequent analysis.

The measurement parameters and methods

The measurements of soil water content, soil available nitrogen content and soil respiration rate: the soil water content was measured with the method of weighting after being dried. The soil available nitrogen content was measured with alkaline hydrolysis diffusion method. Soil respiration rate was measured with EGM-4 portable soil respirator (PP Systems, Amesbury, MA, USA) at each sampling point. Samples at each distance point were collected three times.

Measurement of the activities of soil enzymes: the soil samples within the 0-30 cm layer were collected at the locations of 30, 60, 90 and 120 cm from the edges of the cracks, respectively. The collected samples were fully blended and then put into the ice bottle, immediately brought back to laboratory and stored at -20^{o}C freezer. These soil samples were used for subsequent analysis of the activities of soil enzymes. The urease activity was assayed with sodium phenate colorimetry. The invertase activity was assayed with 3, 5-dinitrosalicylic acid colorimetry. A total of 6 replicate soil samples were measured at each location (Yao et al., 2006).

Measurements of photosynthesis rate and chlorophyll content: the net photosynthesis rate of wheat leaves was measured with LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA) in 9:00-11:00 AM in the morning. Chlorophyll content of the leaves of the same plants was measured with SPAD-502 chlorophyll meter (Minolta Co., Ltd, Osaka, Japan). A total of 6 replicate plant samples were measured at each location.

Measurements of Grain Yield and Yield Trials: At the mature stage of wheat, at each sampling point, the grain yield of wheat at two lines with the length of 1 m were randomly selected to assess the yield capacity. The assessment was repeated three times. At the same time, at each sampling point, a total of 30 wheat plants were selected randomly and used to measure the plant height, weight per stem, number of grain, number of fertile spikelet, the number of infertile spikelet and the 1000-grain weight.

Data were statistically analyzed using Microsoft Excel 2000. The differences in the effects between treatment groups were compared by t-test with $P < 0.05$ being regarded statistically significant.

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Results

Effects of the surface cracks on characteristics of soil water and fertility

The surface cracks caused by mining subsidence not only destroyed soil structure but also led to the loss of soil water and soil fertilizers. During the jointing stage of winter wheat (20 days after the formation of the cracks), the cracks had no significant effects on the available nitrogen content of soil but significantly affected soil water content in the soil surrounding cracks. Cracks with different widths caused the different effects on soil water content. Within 0-30 cm soil layer, C-1 significantly affected the soil water content within 0-60cm from the cracks. When the distance from the cracks was over 60 cm, the cracks had no significant effects on soil water content. The range of soil water content affected by C-2 was only 0-30 cm. Within the soil layer of 30-60 cm, C-2 had no effects on soil water content. C-1 had significant effects on soil water content only within 0-30 cm from the cracks. During the flowering stage, within the soil layer of 0-30 cm, C-1 and C-2 both significantly reduced soil water content and available nitrogen content in the surrounding soil within 0-60 cm and 0-30 cm from the cracks, respectively (*Figs. 3 and 4*). Within the soil layer of 30-60cm, both C-1 and C-2 significantly reduced soil water content and available nitrogen content in the surrounding soil within 0-30 cm from the cracks. When the distance from the cracks was over 30 cm, the cracks had no significant effects on soil water content and available nitrogen content.

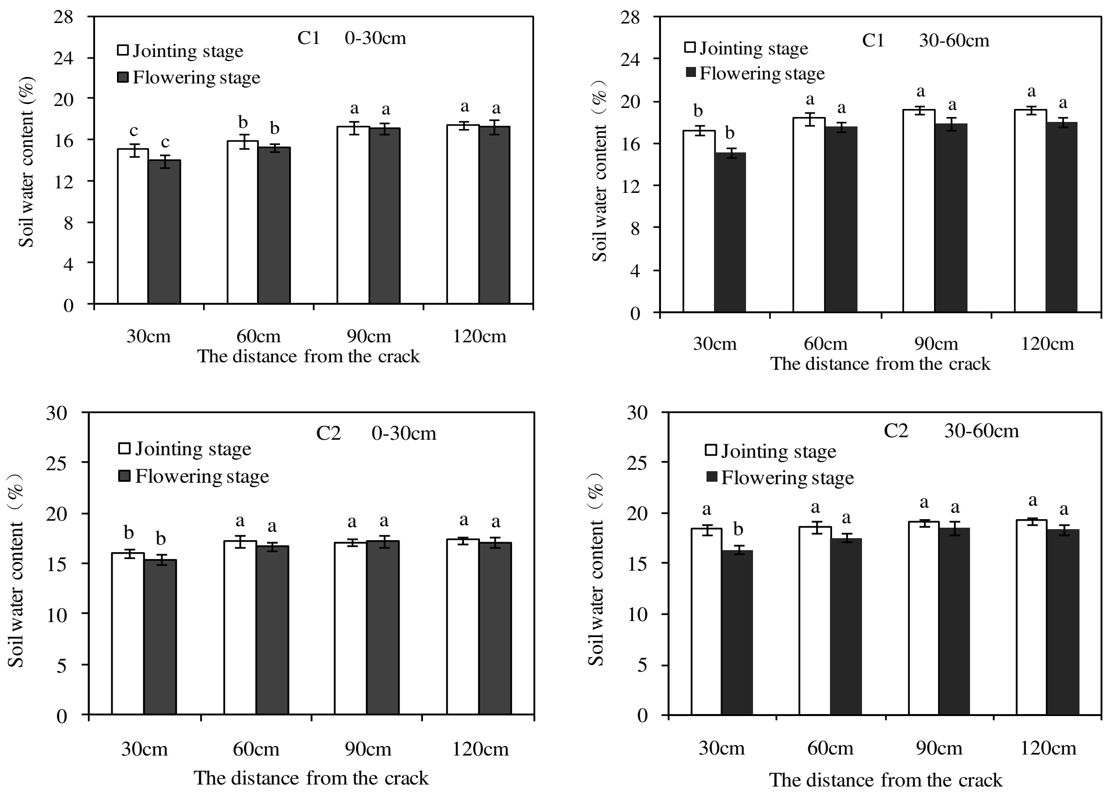

Figure 3. *Effect of surface cracks on soil water content in coal mining subsided area. C-1: larger crack with a 45 m in length, 5-10 cm in width and 200 cm in depth; C-2: smaller crack with a 25 m in length, 2-5 cm in width and 100 cm in depth. Vertical bars represent SE of the mean. For a given growth stage, mean values followed by the same letter did not differ significantly ($p<0.05$).*

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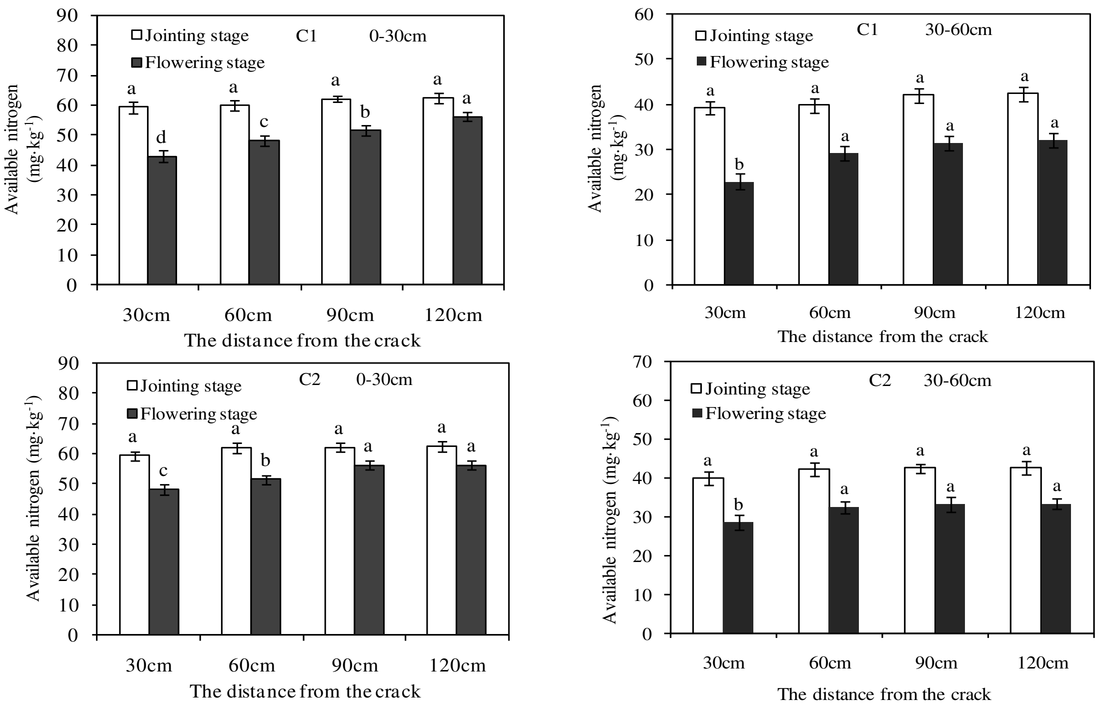

Figure 4. *Effect of surface cracks on available nitrogen content in coal mining subsided area. C-1: larger crack with a 45 m in length, 5-10 cm in width and 200 cm in depth; C-2: smaller crack with a 25 m in length, 2-5 cm in width and 100 cm in depth. Vertical bars represent SE of the mean. For a given growth stage, mean values followed by the same letter did not differ significantly ($p<0.05$).*

Effects of subsided cracks on characteristics of soil microorganisms

The characteristics (such as the activities of soil enzymes and soil respiration rate) of soil microorganisms are very sensitive to the changes in soil environment. Soil respiration rate reflects, in a large extend, the metabolic intensity of soil organic matters and the transformation/supply capacity of soil nutrients. During the jointing and flowering stages, the surface cracks inhibited the respiration rate of the surrounding soil, the closer the soil from the cracks was, the lower the soil respiration rate was. For C-1, the range within which it inhibited soil respiration rate was 0-60 cm. When the distance from the cracks was over 60 cm, its inhibition on soil respiration rate was not significant. The range within which C-2 inhibited soil respiration rate was 0-30 cm. When the distance from the cracks was over 30 cm, its inhibition was no longer significant (*Fig. 5*)

The subsided cracks had significant effects on the activities of both urease and invertase (*Fig. 6*). Within the distance of 0-30 cm from cracks, the cracks caused maximal inhibition on the activities of soil enzymes. With increasing the distance from crack, this inhibition was gradually reduced. However, during different stages, different soil enzymes performed differently. During the jointing stage of winter wheat, C-2 had no significant inhibition on the activities of both urease and invertases. The range within which C-1 caused inhibition on the activities of two enzymes was 0-60 cm. When the distance from the cracks was over 60 cm, its inhibitory effects were no longer significant. During the flowering stage, the ranges within which C-1 and C-2 caused

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inhibition on the activity of inverase were both 0-60 cm. When the distance from the cracks was over 60 cm, their inhibitory effects on invertase were no longer significant. The range within which C-1 caused inhibition on the activity of urease was 0-60 cm while such range for C-2 was 0-30 cm.

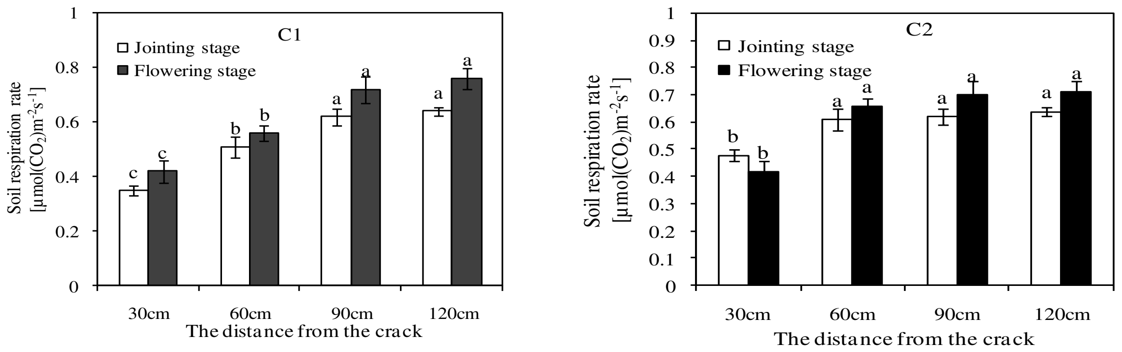

Figure 5. *Effect of surface cracks on soil respiration rate in coal mining subsided area. C-1: larger crack with a 45 m in length, 5-10 cm in width and 200 cm in depth; C-2: smaller crack with a 25 m in length, 2-5 cm in width and 100 cm in depth. Vertical bars represent SE of the mean. For a given growth stage, mean values followed by the same letter did not differ significantly ($p<0.05$).*

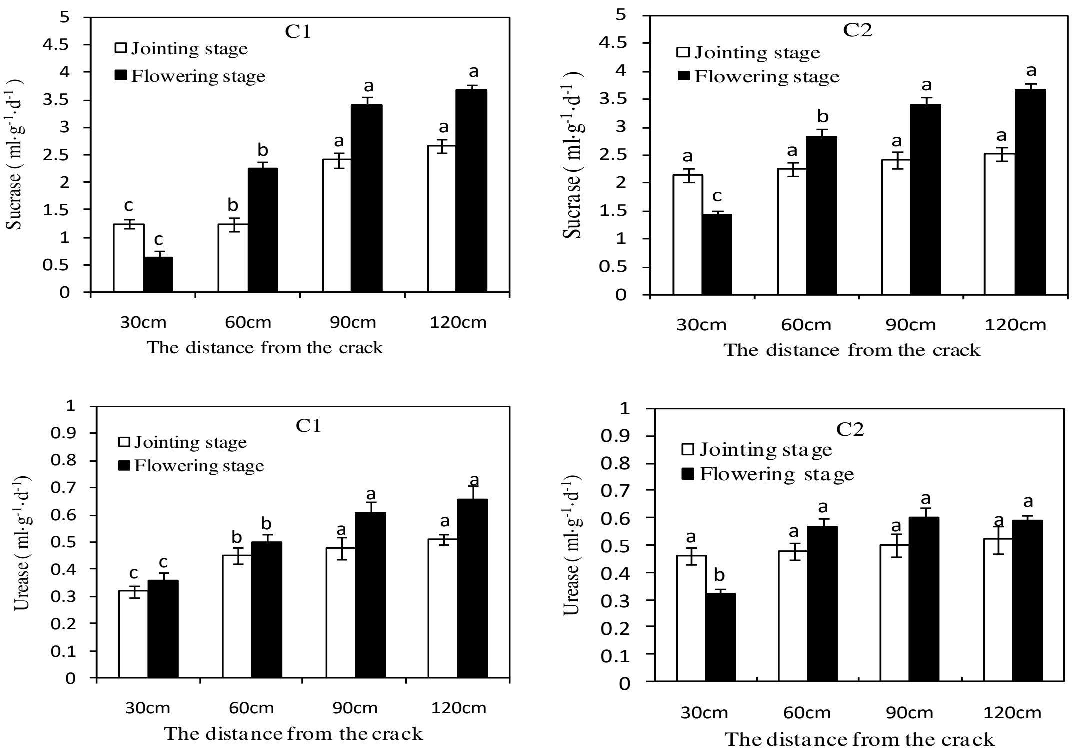

Figure 6. *Effect of surface cracks on soil enzyme activities in coal mining subsided area. C-1: larger crack with a 45 m in length, 5-10 cm in width and 200 cm in depth; C-2: smaller crack with a 25 m in length, 2-5 cm in width and 100 cm in depth. Vertical bars represent SE of the mean. For a given growth stage, mean values followed by the same letter did not differ significantly ($p<0.05$).*

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Effects of subsided cracks on chlorophyll content of wheat leaves and photosynthetic characteristics

Surface cracks affect physiological characteristics of plant via disturbing the physicochemical properties and microbial characteristics of soil. The chlorophyll content of plant leaves and photosynthetic characteristics are the two important physiological characteristics. During jointing and flowering stages, within 30 cm from the crack, the chlorophyll content of wheat leaves was lowest. When the distance from the crack was over 30 cm, the differences in the chlorophyll content of wheat leaves were no longer significant (*Fig. 7*). The photosynthetic rates of wheat leaves were different during different stages. During the jointing stage, both C-1 and C-2 caused significant inhibition on photosynthetic rates within 0-30cm from the crack. When the distance from the crack was over 30 cm, cracks had no significant effects of photosynthetic rate of wheat. During the flowering stage, C-1 caused significant inhibition on photosynthetic rate of wheat within the distance of 0-60 cm from the crack while C-2 caused significant inhibition on photosynthetic rate of wheat leaves within the distance of 0-30 cm from the crack (*Fig. 7*).

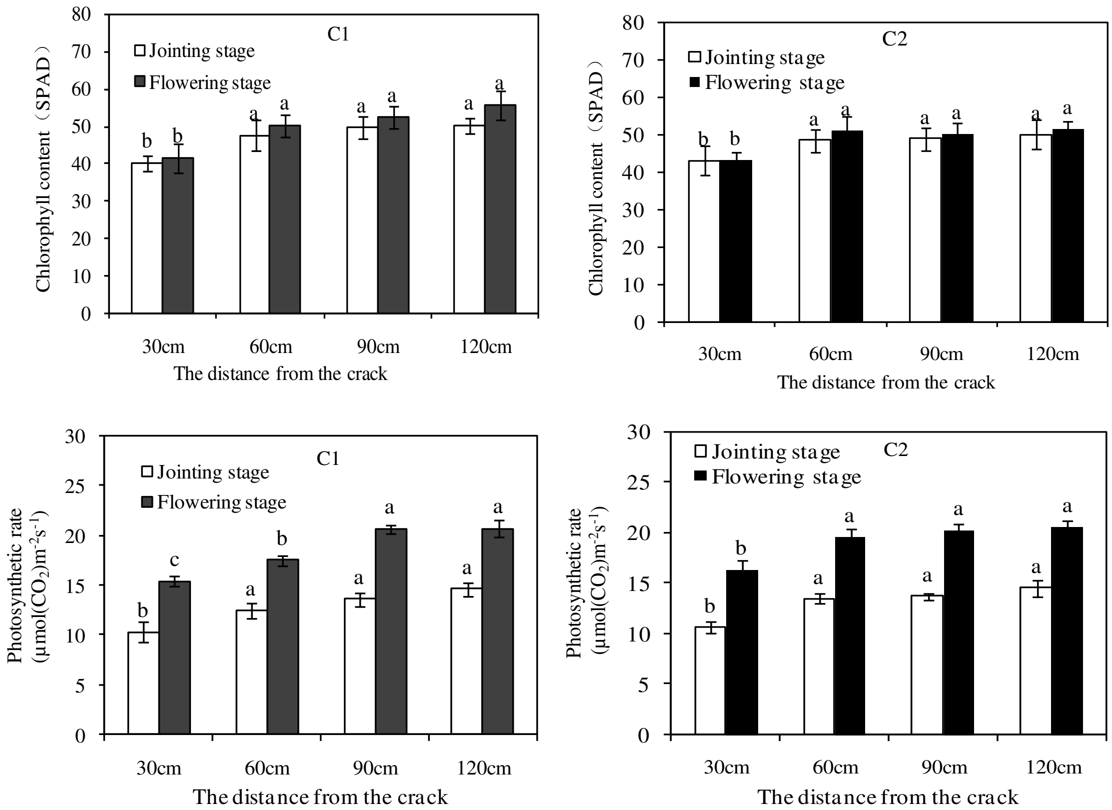

Figure 7. *Effects of the surface cracks on chlorophyll content and photosynthetic rate of wheat in coal mining subsided area. C-1: larger crack with a 45 m in length, 5-10 cm in width and 200 cm in depth; C-2: smaller crack with a 25 m in length, 2-5 cm in width and 100 cm in depth. Vertical bars represent SE of the mean. For a given growth stage, mean values followed by the same letter did not differ significantly (p<0.05).*

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Effects of surface cracks on the yield trail of wheat

It can be seen from *Table 2* that surface cracks had different effects on various yield traits of wheat. Both C-1 and C-2 lowered plant height, weight per stem, and grain number but increased the number of infertile spikelet of wheat within 0-60 cm from cracks. When the distance from the crack was over 90 cm, both C-1 and C-2 had no effects on plant height, weight of single stem, the number of infertile spikelet and the grain number. Both C-1 and C-2 had no significant effects on 1000-grain weight. However, C-1 significantly reduced the number of fertile spikelet and grain yield of wheat within the distance of 0-90 cm from the crack. C-2 significantly reduced the number of fertile spikelet and grain yield of wheat within the distance of 0-60 cm from the crack. Compared with the grain yield of wheat at the distance of 120 cm from cracks, C-1 and C-2 reduced grain yield of wheat at 30 cm from the cracks by 53.3% and 34.8 %, respectively.

Table 2. *Yield traits of wheat in different locations away from the crack (±S.E.)*

Items		Distance from the crack (cm)			
		30	60	90	120
C-1	Plant height(cm)	65.1±1.2c	70.2±1.4b	73.8±1.1a	74.5±1.1a
	Spike number	249.4±9.2d	306.8±4.3c	396.4±5.2b	442.8±8.7a
	Weight per stem (g)	1.12±0.08c	1.56±0.06b	1.72±0.05a	1.76±0.03a
	Infertile spikelet	2.1±0.10a	1.9±0.08b	0.7±0.03c	0.6±0.10c
	Grain number per ear	26.4±1.1c	30.2±1.8b	34.5±1.4a	35.3±1.38a
	Thousand seed weight (g)	36.8±1.1a	37.1±1.3a	37.3±0.9a	36.8±0.6a
	Grain yield($kg \cdot m^{-2}$)	0.21±0.02d	0.36±0.02c	0.39±0.03b	0.45±0.04a
C-2	Plant height(cm)	68.1±1.4c	72.2±1.2b	74.6±1.2a	74.4±1.2a
	Spike number	289.4±9.2c	396.8±4.3b	440.4±4.2a	446.8±7.1a
	Weight per stem (g)	1.32±0.08c	1.66±0.06b	1.71±0.05a	1.74±0.01a
	Infertile spikelet	1.9±0.10a	1.9±0.1a	0.6±0.06b	0.7±0.11b
	Grain number per ear	28.2±1.1c	32.6±1.6b	35.2±1.1a	35.6±1.21a
	Thousand seed weight (g)	36.1±1.1a	37.8±1.0a	37.4±0.4a	37.1±0.4a
	Grain yield($kg \cdot m^{-2}$)	0.28±0.03d	0.35±0.02b	0.44±0.04a	0.46±0.05a

Mean values followed by different small letters in the same line indicate significant difference at 0.05 level.

Discussions

Effects of surface cracks on the characteristics of soil water and fertilizers

Soil cracks cause extremely important effects on the transport process of water and solutes in soil. Coal mining subsidence leads to the formation of cracks (gaps) with different widths on the ground surface, which makes the rainfall to more easily penetrate into underground and thus, reduces the water supply to soil. During the non-raining periods, the crack increases the contact area between soil and external environment and accelerates the lateral evaporation of soil water and exacerbates the loss of water from the soil. Both the reduced water supply and the increased evaporation rate certainly lead to the reduction in soil water content. Furthermore, the vertical cracks (gaps) also weaken the total water-holding capacity of soil. The combined actions of these factors reduce soil water content to different extends (Li et al., 2011). In this study, the result of the investigation on water contents in soil in different spatial

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locations in the subsided area indicates that the cracks significant reduce soil water content in soil surrounding the cracks but the affecting ranges of cracks with different sizes on soil water content varied. The affecting range of the larger crack was larger than that of smaller crack.

The changes in soil nutrients are closely related to the migration of soil water. In the coal mining subsided area, due to the formation of many cracks (gaps) on the ground surface, many nutritional elements in the soil can more easily leak with the direct runoff along cracks during raining, which, in turn, cause the deficiency of soil nutrients, degrade soil environmental quality and seriously affect crop growth (Zhao et al., 2010). In this study, we measured the available nitrogen contents in soils with different distances from the cracks. The results indicated that in the early stage of the crack formation (at the jointing stage of wheat), because of the lack of raining, the available nitrogen content in soil was not changed largely. During the flowering stage, the cracks significantly reduced available nitrogen content in the soil surrounding the cracks. The closer the distance from the crack was, the lower the available nitrogen content in soil was. The effects of the larger cracks on the available nitrogen content in soil were larger than those of the smaller cracks.

Effects of surface cracks on of soil microorganisms

The characteristics of soil microorganisms, including microbial quantity, activities of soil enzymes and soil respiration etc, are very sensitive to the changes in soil ecological quality and are generally used to evaluate the supply capacity of soil nutrients and soil quality (Zhou and Ding, 2007). Du et al. (2013) reported that surface cracks caused by coal mining obviously reduced microbial quantities and activities of soil enzymes, and also altered the compositions and structure of microbial community in the rhizosphere of Artemisia ordosias. Among different indicators reflecting the characteristics of soil microorganisms, soil respiration rate is the most sensitive one to respond the status of soil water and soil nutrients (Singh et al., 1977; Zheng et al., 2014). In this study, subsided cracks reduced the contents of water and available nitrogen in the soils surrounding the cracks and, at the same time, also significantly reduced soil respiration rate.

All the biochemical processes taking place in the soil are carried out and accomplished by soil enzymes (Zhou and Ding, 2007). Soil enzymes are very sensitive to the changes caused by environmental factors and exhibited quite well the characteristics of timeliness. Most of soil enzymes are mainly derived from soil microorganisms. Soil structures and the status of soil water and nutrients all cause important effects on soil microorganisms. The changes in soil physicochemical properties and in the quantitation of soil microorganisms certainly lead to the directional changes in the activities of the soil enzymes (Noah et al., 2003). For instance, the study by Liu et al (2006) indicated that the activities of both soil invertase and urease were highly and positively correlated with the available nitrogen. In this study, the results of the investigation on the activities of invertase and urease in the soils surrounding the cracks also confirmed the above-mentioned results, i.e. the cracks significantly reduced soil available nitrogen content, which, in turn, affected the activities of soil enzymes. Thus, the activities of both invertase and urease in soil were also significantly reduced.

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Effects of surface cracks on physiological characteristics and grain yield trials of wheat

Cultivation of crops requires the high soil quality and the proper landform of farmland. The destroying soil environment will affect the cycling processes of water and nutrients in soil and finally cause serious loss of crop yield (Ma et al., 2014). It has been reported that mine subsidence in Illinois, USA, caused a significantly lower corn yield in subsidence areas (Darmody et al., 1989, 1992, 2014). Surface subsidence and cracks caused by coal mining destroyed the stability and integrality of soil structure. Water and fertilizers easily leak and loss along with the cracks, resulting in increasing degeneration of soil quality. Furthermore, due to the lack of scientific management, proper application of fertilizers and irrigation measures in the subsided area, the poor soil quality is further exaggerated. The contents of water and nutrients in soil are the key factors affecting plant growth. Their levels will directly affect the physiological and ecological characteristics and the yield of crops (Cao et al., 2009). Among which, chlorophyll content of leaves somehow reflects the nitrogen level of plants, which is positively correlated with the soil nitrogen content (Guan et al., 2000). The present study also indicated that changes in chlorophyll content of leaves was consistent with the changes in available nitrogen content in soil, i.e. when the soil was closer to the cracks, the content of the available nitrogen in soil was more significantly reduced and, at the same time, chlorophyll content of wheat leaves was also significantly reduced. These results are the same as those of Xu et al. (2015). Photosynthesis is one of important physiological characteristics of plant and is limited by both the internal factors and external environmental conditions. Soil water and nitrogen are two important environmental factors frequently limiting for plant growth. Water and nitrogen stresses will inhibit photosynthesis and the growth of plant (He et al., 2010). Increasing soil nitrogen content can not only significantly increase chlorophyll content of plant leaves but can also improve the photosynthetic characteristics of crops and increase the grain number and grain weight of wheat (Zhang et al., 2005). In this study, subsided cracks significantly affected the contents of water and available nitrogen in soil and also affected plant’s uptake of trients and water. Thus, plant photosynthesis was also significantly affected. The growth and yield of plant are closely related to the accumulation of photosynthates. The closer to cracks were, the greater the effect on photosynthesis of wheat was. Thus, the plant height, grain number and weight per stem were all significantly reduced. The deficiency in photosynthetic capability finally led to the reduction in grain yield of crop. A recent study by Hu et al.indicated that in the eastern plains of China, the overlapping of coal resources and cropland occupied a total area of 1.33×10^5 km^2, accounting for 31.93% of the total cropland area. It is estimated that by 2020, the accumulative total area of cropland destroyed by continuing coal mining in that area may reach as high as 3.83×10^3 km^2, leading to the reduction of grain yield by 9.63×10^8 kg and an increase in number of landless farmers up to 1.91×10^6 (Hu et al., 2014a). Similar situation may also happen in coal mining areas in Henan province. Therefore, further studies are needed to address this problem and to find effective measures to resolve it.

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Conclusions

The effect of surface cracks caused by coal mining on the soil characteristics and crop growth was studied in subsided area of Zhaogu coal mine of Jiaozuo Coal Group, China. The results show that surface cracks affected significantly soil characteristics in the soil surrounding the cracks. The larger crack (C-1) had larger effect ranges on both soil water content and the characteristics of soil microorganisms compared to smaller crack (C-2). Surface cracks lowered significantly chlorophyll content and photosynthetic rate of plant via affecting soil characteristics. During jointing and flowing stage, both C-1 and C-2 caused significant inhibition on photosynthesis of wheat within 0-60 cm and 0-30cm from the cracks, respectively. Surface cracks promoted loss of water and nutrients in the soil surrounding the cracks, which finally led to significant reduction in crop yield. The large crack had stronger effects on grain yield compared to small crack. Therefore, further studies are needed to address this problem and to find effective measures to resolve it.

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EFFECT OF SUPERABSORBENT POLYMER ON SALT AND DROUGHT RESISTANCE OF *EUCALYPTUS GLOBULUS*

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Abstract. In this study, the effect of Super-AB-A-200 polymer on salt and drought resistance of *Eucalyptus globulus* Labill. was evaluated. The treatments were: (1) Control, Control + polymer; (2) NaCl, NaCl+ polymer; (3) Drought, Drought + polymer; (4) Drought + NaCl, Drought + NaCl + polymer. The results showed that Super-AB-A-200 in root medium helped *E. globulus* cuttings to resist the salinity and drought stresses, because of the following reasons: (1) *E. globulus* roots absorbed the held water from Super-AB-A-200 polymer in soil water-deficit conditions; (2) in saline conditions, Super-AB-A-200 retained Cl^- and Na^+ in the soil solution because of their high water-holding capability, moreover, the exchangeable K^+ included in Super-AB-A-200 resulted in an amended K^+/Na^+ balance in salinized plants; (3) Super-AB-A-200 helped *E. globulus* cuttings to resist interactive effects of salinity and drought stresses, which was essentially justified by their salt- and water-holding capabilities.
Keywords: *acrylate polymers, gas-exchange, salinity stress, water-saving agriculture, water use efficiency*

Introduction

A lot of countries have not sufficient water resources to confront their actual environmental, urban and agricultural requirements. With increasing water deficiency, population and water claims are increasing simultaneously (Bouwer, 2002). Superabsorbent polymer (SAP) perform as a soil reformer to decrease soil water loss and increase crop yield. SAP is a hydrophilic polymer that can store and take up 1000 times more water or aquatic solutions than its first weight and size (Sojka and Entry, 2000). Therefore, SAP can increase soil water-holding capacity and nutrient use efficiency (Lentz et al., 1998) and decrease water loss (Al-Omran and Al-Harbi, 1997). SAP is applied in the soil to make a water supply, near the rhizosphere zone and advantage agriculture (Han et al., 2010). Because of the water resource crisis, water-saving agriculture is necessary for the sustainable development. Moreover, droughts are anticipated to be increasingly crucial because of climate change (Gornall et al., 2010). Hydrophilic polymers are usually cross-linked 3-D hydrophilic nets that are able to take up and keep noteworthy values of aquatic liquids even in certain pressure or temperature. SAPs are able to perform in several fields like agriculture (Puoci et al., 2008), sanitary productions (Kosemund et al., 2009), waste water refining (Kasgoz and Durmus, 2008; Kasgoz et al., 2008; Wang et al., 2008a). SAPs are in three types containing natural, semi-artificial and artificial (Mikkelsen, 1999). Unnatural polymers applied more than natural ones because they have more endurance against environmental collapse (Peterson, 2002). Superabsorbent minimizes micronutrients from washing out to the water table and causes more water use efficiency, a decline in costs of irrigation and intervals by 50 percent water stress and damages to transplant during transferring (Abedi Koupai and Mesforoush, 2009). Teimouri and Sharifan (2013) assessed the effect of KCl and NaCl in varied concentrations on dehydration and hydration of some SAPs. According to the results, Super-AB-A-200 and Colophony had

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the most hydration and dehydration respectively. Fazeli-Rostampoor et al. (2011) showed that drought stress and applying Super-AB-A-200 had a significant effect on increasing corn grain yield and water use efficiency. Li et al. (2014) reported that the addition of SAPs significantly increased the soil water content and soil maximum hygroscopic moisture in the booting and filling stages but had no effects on the soil available water-holding capacity compared with the control in the filling stage. Yadollahi et al. (2012) assessed the impact of Super-AB-A-200 and organic matters in soil water retention and construction of almond orchards in rainfed conditions. The results showed that Super-AB-A-200 and organic matters could increase soil water retention significantly. Besides, this conditions could increase growth indices of almond seedlings. *Eucalyptus globulus* is a woodland tree and native to Australia, however, it has been planted in Iran and is under evaluation. In spite of ecological importance, Eucalyptus has important medicinal and horticultural worth and several species are applied for forest landscape and street planting. The *E. globulus* trees are used in the south of Iran as park landscape and street planting. Besides, salinity and drought stresses are essential restrictions for their survival. SAPs may help them to tolerate these harmful stresses. The aim of this study was to evaluate the effects of Super-AB-A-200 polymer on salt and drought resistance in *Eucalyptus globulus* Labill.

Materials and Methods

Hydrophilic polymer

The hydrophilic polymer Super-AB-A-200, produced by Rahab Resin Co. with product license holding of Iran Polymer and Petrochemical Institute (Rahab Resin Co, 2016), was used in this study (*Table 1*). This hydrophilic polymer is a granular-type tripolymer of acrylamide, acrylic acid, and acrylate potassium.

Table 1. *The characteristics of Super-AB-A-200 polymer*

Characteristics	Super-AB-A-200 polymer
Shape	Granular
Density	1.4-1.5 (gr.cm^{-3})
Size of particles	50-150 (µm)
Maximum stability in soil	7 (year)
Practical capacity of water uptake	220 (g.g^{-1})

Experimental treatments

One-year-old cuttings of *Eucalyptus globulus* (48 plants) were obtained from a nursery under natural conditions near Shush, Iran. The cuttings were planted separately in 6-L pots filled with sandy soil. Prior to treatment, the plants were grown in a greenhouse and well-irrigated with 1000 mL of Hoagland's nutrient solution every 15 days. Uniform plants with a height of about 90 cm were collected for this study. The plants were transferred to 15-L pots randomly filled with either saline soil (sandy soil pretreated with 2000 mL of 300 mM NaCl) or a control soil without the NaCl with the polymer or without it (0.5% of dry weight). The four treatment groups were classified as follows: (1) Control + Super-AB-A-200, Control; (2) NaCl + Super-AB-A-200, NaCl;

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(3) Drought + Super-AB-A-200, Drought; (4) NaCl + Drought + Super-AB-A-200, NaCl + Drought. The drought treatment was applied after the start of water-deficit stress by withholding irrigation, but the control plants were well-irrigated.

Plant harvesting

The experimental plants were harvested (three replicate plants for each treatment) after 40 days of exposure to drought and salinity treatments. Plant roots were thoroughly washed with deionized water to remove soil. The fresh weights of the leaves, roots, and stems were assessed and the tissues were dried in an oven at 65°C for 5–7 days to determine the dry weight. Dried samples were ground into powder and stored for analysis.

Plant water usage capacity

Each pot containing one plant was covered by a plastic case and protected at the stem base to stop rainfall and soil evaporation. The water usage was measured as the daily weight loss of the pot and the plant together over a 12-h period (08:00–20:00) on days 4, 8, 12, 16, and 20 and the mean value of each plant was recorded. Three individual replicates were examined for each treatment.

Gas-exchange of leaves

The gas-exchange of the 5th through 7th leaves of the *E. globulus* shoots was determined on days 17, 28, 34, 36, and 38 and the mean value of each plant was recorded (the radiation of natural light was greater than 600 µmol m^{-2} s^{-1}). Stomatal conductivity (Gs), net photosynthetic rates (Pn), and leaf transpiration rates (TRN) were recorded between 9:00–10:30 a.m. with a CIRAS-3 Portable Photosynthesis System (PP Systems, Amesbury, MA, USA) where the photosynthetically active radiation (PAR) was approximately 1300 µmol m^{-2} s^{-1}. If the PAR was very low, the light was supplemented by halogen lamps (Shi et al., 2010). Leaf temperatures were 30 to 35°C during the measurements.

Ion analysis of roots and leaves

Ion analysis was conducted according to Storey (1995). Dried samples (1 g) of roots and leaves were extracted with 2 $NHNO_3$. K^+ and Na^+ were measured by an atomic absorption spectrometer (AAS) (PerkinElmer 2380). PerkinElmer 2380 is a double-beam, optical system and high-dispersion monochromator with a wavelength range of 190 to 870 nm. Cl^- was measured by an adjusted method of silver titration. A solution of $AgNO_3$ (0.027 N) was applied to the sediment the Cl^- of aquatic extracts and surplus, Ag^+ was evaluated by NH_4SCN (0.01 N) titration. $NH_4Fe(SO_4)_2$ was applied as a color indicator for ionic spot assignment. Concentrations of Cl^- were determined by the equation (1) as follows (Shi et al., 2010):

$$Cl^- \, (\text{mmol g}^{-1}\text{ DW}) = \frac{N_{AgNO_3} V_1 - N_{NH_4SCN} V_2}{DW} \quad \text{(Eq.1)}$$

where
DW: dry weight (g)
V_1: total volume of the $AgNO_3$ solution in chloride extracts (mL),
V_2: volume of the NH_4SCN solution, applied for surplus Ag^+ sediment (mL).

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Soil ion analysis

The soil was sampled at harvest and the soil water content was tested. The soil sample extracts (dried soil:deionized water = 1:5, w/v) were used to measure Cl^-, K^+, and Na^+ contents. K^+ and Na^+ were measured by AAS (PerkinElmer 2380) at 771 and 594 nm, respectively; and Cl^- was measured by silver titration.

The analysis of data

ANOVA was used to compare the data and significant differences between mean values were measured by Duncan's multiple range test. Differences between means were designated as statistically significant when $p < 0.05$.

Results

Salinity and drought stress-induced leaf damages

Leaf damage gradually increased as the soil water deficit increased and about 40% of *E. globulus* leaves were withered after 40 days of drought treatment (*Figure 1*).

Figure 1. *The withered leaves of E. globulus*

Dry weight of plant

The dry weights of the *E. globulus* stems, roots, leaves, and whole plants were significantly reduced under salinity and/or drought stresses compared to the control. However, the NaCl + Drought treatment did not decrease dry weight more than the salt and drought stresses alone (*Table 2*). The plants treated with the Super-AB-A-200 polymer had higher dry weights compared to the plants without the polymer in all treatments (*Table 2*). The stem, leaf, and whole plant dry weights in the NaCl + Drought + polymer treatment were not significantly different from the control. This indicated that the Super-AB-A-200 polymer improved growth under salinity stress, especially in

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drought conditions. In addition, the Super-AB-A-200 polymer significantly improved the dry weights of the *E. globulus* stems, roots, leaves, and whole plants in the NaCl, drought, and NaCl + Drought treatments (*Table 2*).

Table 2. *The effect of Super-AB-A-200 polymer on dry weight of stems, roots, leaves and whole plants of Eucalyptus globulus under NaCl and/or drought stresses*

Dry weight (g)	Treatment	- Polymer	+ Super-AB-A-200
Root	Control	15.23a^{A}	15.78a^{A}
	NaCl	10.42b^{B}	12.71a^{B}
	Drought	10.63b^{B}	12.48a^{B}
	NaCl + Drought	10.21b^{B}	12.41a^{B}
Leaf	Control	16.25a^{A}	16.51a^{A}
	NaCl	11.48b^{B}	13.69a^{B}
	Drought	11.68b^{B}	12.67a^{B}
	NaCl + Drought	11.31b^{B}	14.98a^{B}
Stem	Control	16.49a^{A}	16.41a^{A}
	NaCl	11.45b^{B}	13.21a^{B}
	Drought	11.74b^{B}	12.88a^{B}
	NaCl + Drought	11.39b^{B}	14.17a^{B}
Plant	Control	52.56a^{A}	53.88a^{A}
	NaCl	39.61b^{B}	45.56a^{B}
	Drought	40.43b^{B}	44.14a^{B}
	NaCl + Drought	39.12b^{B}	47.68a^{B}

Every value is the average of three separate plants. Values pursued by varied letters in the similar column (A, B) or the similar row (a, b) are significantly varied at $p<0.05$.

Plant water use efficiency

The daily water use of the *E. globulus* cuttings was reduced under salinity or drought stress in the presence of the polymer, and this decline was further evident in the NaCl + Drought treatment (*Table 3*).

Table 3. *The effect of Super-AB-A-200 polymer on plant water use (gH_2Oday^{-1}) in Eucalyptus globulus plants under NaCl and/or drought stresses*

Treatment	- Polymer	+ Super-AB-A-200
Control	164.5a^{A}	168.6a^{AB}
NaCl	141.8b^{B}	165.9a^{AB}
Drought	129.4b^{B}	153.1a^{B}
NaCl + Drought	95.7b^{C}	177.6a^{A}

Every value is the average of three separate plants. Values pursued by varied letters in the similar column (A, B, C) or the similar row (a, b) are significantly varied at $p<0.05$.

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Gas-exchange of leaves

The transpiration rates (TRN), net photosynthetic rates (Pn), and leaf stomatal conductivity (Gs) of the *E. globulus* cuttings was reduced over time in the salinity or drought stress treatments (*Table 4*). In the NaCl + Drought treatment the TRN, Pn, and Gs was reduced by 30%, 48%, and 55%, respectively (*Table 4*). The addition of Super-AB-A-200 increased the gas-exchange in the NaCl-, Drought-, and NaCl + Drought-treated plants (*Table 4*). The effect of the Super-AB-A-200 on the gas-exchange in the drought-stressed plants was significantly higher than the NaCl-treated plants (*Table 4*).

Table 4. *The effect of Super-AB-A-200 polymer on transpiration rates (TRN), net photosynthetic rates (Pn) and leaf stomatal conductivity (Gs) in Eucalyptus globulus plants under NaCl and/or drought stresses*

Gas-exchange	Treatment	- Polymer	+ Super-AB-A-200
Gs (mmol m^{-2} s^{-1})	Control	138.56a^{A}	144.32a^{A}
	NaCl	95.22b^{B}	113.76a^{B}
	Drought	96.51b^{B}	168.58a^{A}
	NaCl + Drought	62.49b^{C}	130.28a^{AB}
TRN (mmol m^{-2} s^{-1})	Control	4.21a^{A}	4.45a^{AB}
	NaCl	3.51b^{B}	4.18a^{B}
	Drought	3.97b^{AB}	5.15a^{A}
	NaCl + Drought	2.96b^{C}	4.49a^{AB}
Pn (µmol m^{-2} s^{-1})	Control	14.12a^{A}	14.85a^{A}
	NaCl	10.65b^{B}	12.35a^{B}
	Drought	10.75b^{B}	14.30a^{A}
	NaCl + Drought	7.35b^{C}	11.91a^{B}

Every value is the average of three separate plants. Values pursued by varied letters in the similar column (A, B, C) or the similar row (a, b) are significantly varied at $p<0.05$.

Ion concentrations in roots and leaves

The concentrations of Cl^- and Na^+ in the leaves and roots of *E. globulus* significantly increased after 40 days of NaCl treatment compared to the control and the drought stress aggravated the increased ion concentrations in salinized plants, particularly Cl^- (*Table 5*). Application of Super-AB-A-200 polymer decreased the accumulation of Cl^- and Na^+ in leaves and roots in the presence or absence of drought stress and could confine Cl^- and Na^+ in NaCl + Drought-treated plant organs (*Table 5*). The Super-AB-A-200 polymer did not significantly affect the concentrations of Cl^- and Na^+ in the leaves and roots under drought stress conditions, with some exceptions (*Table 5*). The NaCl treatment decreased the concentration of K^+ in the roots irrespective of drought treatment, but this did not occur in the leaves (*Table 5*). Moreover, the addition of Super-AB-A-200 altered K^+ in leaves and roots of drought-stressed plants (*Table 5*).

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Table 5. *The effect of Super-AB-A-200 polymer on K^+, Cl^- and Na^+ concentrations in roots and leaves of Eucalyptus globulus plants under NaCl and/or drought stresses*

Ion concentration	Treatment	- Polymer	+ Super-AB-A-200
Leaf			
Na^+ (mM)	Control	0.019a^B	0.019a^B
	NaCl	0.074a^A	0.048b^A
	Drought	0.033a^B	0.043a^A
	NaCl + Drought	0.078a^A	0.025b^B
Cl^- (mM)	Control	0.105a^C	0.098a^B
	NaCl	0.211a^A	0.145b^A
	Drought	0.171a^B	0.178a^A
	NaCl + Drought	0.235a^A	0.065b^B
K^+ (mM)	Control	0.022b^A	0.101a^A
	NaCl	0.020b^A	0.105a^A
	Drought	0.024b^A	0.127a^A
	NaCl + Drought	0.026b^A	0.113a^A
Root			
Na^+ (mM)	Control	0.058a^B	0.060a^A
	NaCl	0.101a^A	0.076b^A
	Drought	0.061a^B	0.085a^A
	NaCl + Drought	0.102a^A	0.063b^A
Cl^- (mM)	Control	0.112a^C	0.108a^A
	NaCl	0.215a^B	0.087b^A
	Drought	0.181a^B	0.074b^A
	NaCl + Drought	0.356a^A	0.106b^A
K^+ (mM)	Control	0.415b^A	2.05a^A
	NaCl	0.295b^B	2.66a^A
	Drought	0.404b^A	1.97a^A
	NaCl + Drought	0.331b^B	2.51a^A

Every value is the average of three separate plants. Values pursued by varied letters in the similar column (A, B, C) or the similar row (a, b) are significantly varied at $p<0.05$.

Ion concentrations in soils

As expected, the concentrations of Cl^- and Na^+ increased significantly in the NaCl-treated soils compared to the control soils, and drought stress increased the soil salinity (*Table 6*). However, addition of the Super-AB-A-200 polymer reduced the Cl^- and Na^+ levels in the saline soil in both the NaCl and the NaCl + Drought treatments (*Table 6*). The Super-AB-A-200 treatments increased the K^+ concentration in the NaCl-treated soil (*Table 6*). The K^+ concentration in the drought-treated soil was reduced by application of Super-AB-A-200; however, application of Super-AB-A-200 did not decrease the K^+ level in NaCl + Drought-treated soil (*Table 6*).

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Table 6. *The effect of Super-AB-A-200 polymer on K^+, Cl^- and Na^+ concentration in soil under salinity and/or drought treatments*

Ion concentration	Treatment	- Polymer	+ Super-AB-A-200
Na^+ (mM)	Control	8.32a^C	8.15a^B
	NaCl	64.35a^B	41.52b^A
	Drought	19.26a^C	11.84b^B
	NaCl + Drought	95.12a^A	21.46b^A
Cl^- (mM)	Control	45.61a^C	47.55a^B
	NaCl	162.33a^B	104.66b^A
	Drought	180.22a^B	78.45b^A
	NaCl + Drought	322.76a^A	59.64b^B
K^+ (mM)	Control	3.01b^B	6.38a^A
	NaCl	3.05b^B	10.66a^A
	Drought	7.53a^A	6.52a^A
	NaCl + Drought	9.06a^A	8.47a^A

Every value is the average of three separate plants. Values pursued by varied letters in the similar column (A, B, C) or the similar row (a, b) are significantly varied at $p<0.05$.

Discussion

The effect of Super-AB-A-200 polymer on drought tolerance

The Super-AB-A-200 polymer improved the growth in the *E. globulus* cuttings (*Table 2*) and reduced the occurrence of leaf damage caused by the drought treatments. These results are similar to other studies, in which the application of SAPs improved the growth of *Citrus* (Arbona et al., 2005), *Eucalyptus* (Viero and Little, 2006) and *Populus popularis* (Shi et al., 2010) in water-deficit conditions. Shi et al. (2010) reported that the application of SAPs improved the growth of *P. popularis* cuttings. The effects of SAPs result from the excess water retained by the SAP granules (Bouranis et al., 1995). Super-AB-A-200 polymer is a tripolymer of acrylamide, acrylic acid, and acrylate potassium. This hydrophilic polymer has tridimensional hydrophilic networks that can absorb and hold a large volume of water equal to hundreds of times its own weight (Abedi-Koupai and Asad-Kazemi, 2006; Marandi et al., 2009). Consequently, *E. globulus* roots absorb water held by the polymer. According to our results, *E. globulus* roots accumulated around the Super-AB-A-200 granules, rather than growing inside them. This result is consistent with the observation that drought-treated plants had fewer leaves, reduced gas-exchange and decreased plant water use without the polymer (*Tables 4 and 3*). Thus, the Super-AB-A-200 polymer lengthened the period of water supply for the plant.

The effect of Super-AB-A-200 polymer on salt tolerance

E. globulus is a semi-salt-tolerant species with a medium capacity for salt exclusion (Osareh and Shariat, 2009). Accumulation of salts in plant cells can cause toxicity and oxidative injury (Wang et al., 2007, 2008b). In this study, the increase of plant growth

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and leaf gas-exchange in the polymer-treated *E. globulus* plants is likely due to its increased capability for salt exclusion. When amended with the Super-AB-A-200 polymer, the Cl^- and Na^+ concentrations were reduced in the leaves and roots of the plants under NaCl stress (*Table 5*). Application of Super-AB-A-200 polymer also reduced the concentration of salt in the soil water solution because of its salt-holding capability. Thus, minimal amounts of salts were taken up by the roots (*Table 5*). Furthermore, the concentrations of the Cl^- and Na^+ in the Super-AB-A-200 polymer were diluted because of the water volume held in the polymer. The concentration of Na^+ was 0.005 mM and the concentration of Cl^- was 0.004 mM in Super-AB-A-200. Thus, root accumulation in or around the polymer resulted in less salt taken up by the plant roots, resulting in improved plant growth and gas-exchange under salinity stress (*Tables 2–4*). Moreover, the Super-AB-A-200 decreased the Na^+ concentration in the roots and leaves of the plants under NaCl stress (*Table 5*) due to the increased exchangeable K^+ level in the Super-AB-A-200 polymer (*Table 6*), thereby improving salt exclusion capability of the plants. This result was in accordance with Shi et al. (2010) in which K^+ levels in *P. popularis* roots and leaves were increased by amendment with SAPs of Stockosorb and Luquasorb on saline soil. Additionally, the Super-AB-A-200 polymer provided a K^+ source, consequently, uptake of K^+ increased in the plants treated with the polymer. K^+ enrichment led to a K^+ balance in the plants under salt stress, resulting in enhanced salt resistance, since the balance of K^+/Na^+ is pivotal for tolerance to toxicity from ions (Munns and Tester, 2008; Sun et al., 2009a,b; Shabala and Cuin, 2008; Shi et al., 2010). Our results showed that the addition of Super-AB-A-200 polymer can improve access to quality water and a source of K^+ while decreasing contact with Cl^- and Na^+, thereby promoting resistance to salt stress.

The effect of Super-AB-A-200 polymer on salt and drought tolerance

The water consumption of NaCl + Drought-treated plants was reduced by 42%, which was significantly greater than the plants under drought or salt stress alone, 21–14% (*Table 3*). Leaf gas-exchange showed a similar trend (*Table 4*). This was the consequence of an interaction of salinity and drought stresses. As the soil dried, the water availability decreased and the concentration of salt ions in the soil increased (*Table 6*). The roots could not absorb enough water to compensate for the water lost through the shoots, resulting in leaf damage. The impact of the co-existing drought and salinity stresses on dry weight was not as apparent as effect on short-term factors such as daily plant transpiration and leaf gas-exchange (*Tables 2–4*). There are three possible explanations as to why the Super-AB-A-200 polymer improved plant performance under drought and salinity stress in this study: (1) the water-filled Super-AB-A-200 polymer granules increased the access to water (*Tables 4 and 3*), (2) the exchangeable K^+ in this polymer promoted K^+/Na^+ balance (*Table 5*), and (3) the polymer granules retained the salt ions, maintaining a lower concentration in the drying soil (*Table 6*). It is important to note that the effects of Super-AB-A-200 on plant dry weight and leaf gas-exchange were considerable (*Tables 2–4*). The addition of Super-AB-A-200 lengthened the duration of water supply to the plants and consequently decreased the damage caused by high soil Cl^- and Na^+ in drought and salinity conditions (*Table 6*). As a result, the absorption of salts by roots and transportation of salt from the root to shoot were both effectively limited, resulting in improved plant growth and survival during an extended period of drought and salinity stress.

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Conclusion

According to the results, Super-AB-A-200 polymer could store water and nutrients in the sandy soil and release them under drought stress conditions. Besides, it could improve sandy soils and increase water-holding capacity. In addition, in saline conditions, Super-AB-A-200 retained Cl^- and Na^+ in the soil solution because of their high water-holding capability and the exchangeable K^+ included in Super-AB-A-200 resulted in an amended K^+/Na^+ balance in salinized plants. Furthermore, Super-AB-A-200 helped *E. globulus* cuttings to resist interactive effects of salinity and drought stresses, which was essentially justified by their salt- and water-holding capabilities. Finally, it is recommended to use other types of hydrophilic polymers for the cultivation of other plants because of their effects on the other ions (heavy metals, calcium and etc). In addition, it is recommended to use Super-AB-A-200 polymer for the cultivation of other plants especially in arid and semi-arid regions with sandy soil. Because this polymer significantly improved the dry weights of the *E. globulus* stems, roots, leaves, and whole plants in the NaCl, drought, and NaCl + Drought treatments, decreased the accumulation of Cl^- and Na^+ in leaves and roots in the presence or absence of drought stress and could confine Cl^- and Na^+ in NaCl + Drought-treated plant organs, reduced the Cl^- and Na^+ levels in the saline soil in both the NaCl and the NaCl + Drought treatments and increased the K^+ concentration in the NaCl-treated soil. Therefore, by using this polymer, lower quality and amount of water could be used for cultivation and the cultivated lands could be extended by storing water in reservoirs.

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VARIABILITY OF PARTICULATE MATTER PM10 CONCENTRATION IN SOSNOWIEC, POLAND, DEPENDING ON THE TYPE OF ATMOSPHERIC CIRCULATION

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Abstract. The aim of the study was to evaluate variation in PM10 concentrations in the city of Sosnowiec, Poland in relation to the type of atmospheric circulation. Data on the average daily concentration of PM10 during the heating season in the years 2013-2015 were obtained from the Sosnowiec monitoring station on Lubelska Street, belonging to the Air Quality Monitoring System run by the Voivodeship Inspectorate for Environmental Protection in Katowice. PM10 concentrations were found to be higher in anticyclonic conditions, on average 55 $\mu g/m^3$, as compared to 45 $\mu g/m^3$ in cyclonic conditions. The highest risk of exceeding the permissible PM10 concentration is observed in Ka, Sa, SWa and Ca conditions. The results of this study may be used to predict high PM10 concentrations and exceedances of permissible levels based on synoptic forecasts.
Keywords: *air quality, particulate matter, heating seasons, air masses advection, baric system*

Introduction

Air pollution is one of the most important environmental issues. Poland has exceptionally poor air quality, which is largely due to excessive PM10 particulate matter concentrations. According to the European Environment Agency, Poland ranks second in Europe in terms of the daily concentration of PM10 in the air (European Environment Agency, 2016). Analysis of spatial variations in pollutant concentrations within Poland indicates that they are higher in the southern part of the country, especially in the Silesia and in Małopolska regions. Particularly high air pollution occurs in the Upper Silesian Agglomeration, which is linked to the industrial character of this area, dating back many years, as well as its high population density (WIOŚ Katowice, 2016). Air pollution negatively affects human health. Particulates in the air can increase the incidence of respiratory and cardiovascular diseases and the risk of cancer, and affects life expectancy. Periods with high concentrations of pollutants, known as smog, are particularly dangerous. The level of pollutants in the air is affected not only by current emissions associated with the municipal and housing sector, transport, and industry, but also by local and regional meteorological conditions, such as air temperature, precipitation, wind velocity, and the type of atmospheric circulation (Majewski, 2005; Czarnecka and Kalbarczyk, 2008; Leśniok and Caputa, 2009, Leśniok et al. 2010). The focus of the present work was the concentration of PM10 particulate matter in the city of Sosnowiec, located within the Zagłębie Dąbrowskie (coal basin), which is part of the Silesian Voivodship (region). In terms of the number of inhabitants, Sosnowiec is the third largest city in Silesia and fifteenth largest in the whole country. The aim of the present study was to assess the variability of PM10 concentrations in the area of Sosnowiec, depending on the type of atmospheric circulation.

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Materials and methods

The basis for the study consisted in the data on average daily concentrations of PM10 particulate matter in the months of the heating seasons, within the period of 2013-2015. Exceedances of the permissible concentrations of PM10 in the air (Official Journal of Laws 2012 item 1031) are closely linked to the combustion of fossil fuels of poor quality in home furnaces and fireplaces (WIOŚ Katowice 2014). Air pollution is characterized by seasonality (Majewski, 2007; Czarnecka and Nidzgorska-Lencewicz, 2010). The concentrations analysed were recorded at the monitoring station in Sosnowiec on Lubelska street (*Fig. 1*), belonging to the Air Quality Monitoring System run by the Regional Inspectorate for Environmental Protection in Katowice.

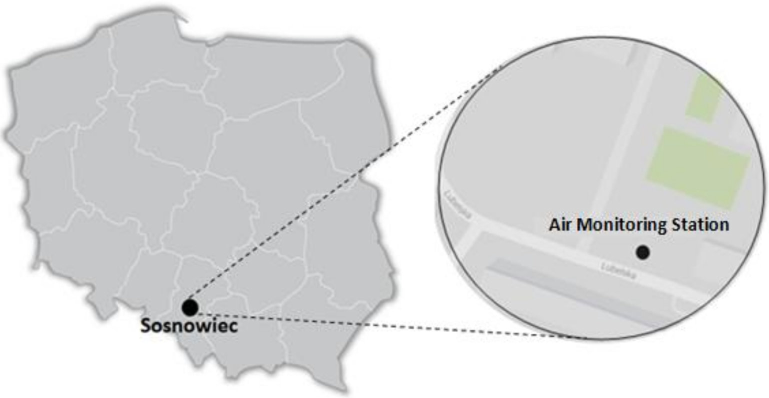

Figure 1. Location of the air quality monitoring station

The calendar of circulation types developed by Niedźwiedź (Niedźwiedź, 1981) was used to analyse the concentrations of PM10 particulate matter depending on the type of atmospheric circulation. For each of the 21 types of atmospheric circulation, the incidence and the mean, maximum and minimum concentrations of PM10 were recorded during the heating season months, i.e. January, February, March, April, October, November and December in the period of 2013-2015, for a total number of 636 days. The overall incidence of each atmospheric circulation type and their frequency of occurrence on days with excessive PM10 levels have been presented.

Research results

Using the data from the measurement station belonging to the Regional Inspectorate for Environmental Protection in Katowice, the average daily concentration of PM10 particulate matter was computed and assigned to each day of the heating season. The arithmetic mean of the PM10 concentration reached 50.9 µg/m^3, the standard deviation was 29.6 µg/m^3, and the variation coefficient was 58.0%, which means that the PM10 concentration varied considerably during the study period. The analysis showed that the permissible levels were exceeded 252 times, which represents 40.0% of the total period under consideration (*Fig. 2*).

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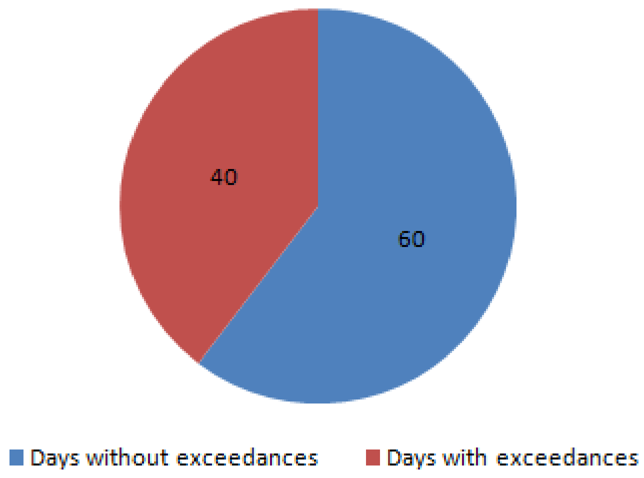

Figure 2. *Percentage share of days with exceedances of permissible concentrations of PM10, as well as days without exceedances throughout the study period*

It should be noted that, as required by law, the permissible annual number of days with exceeded permissible levels of airborne particulate matter is 35 (Official Journal of Laws 2012 item 1031), while in Sosnowiec only during the heating seasons in the years 2013, 2014, and 2015, the number of days exceeding the air quality standards was 106, 77 and 66, respectively, and thus far in excess of the permissible value of pollution (*Fig. 3*).

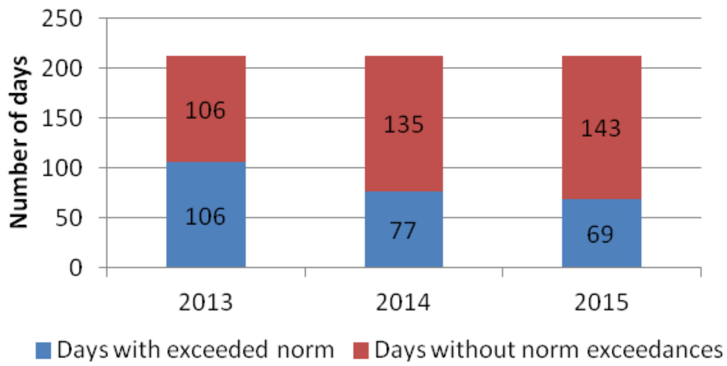

Figure 3. *Number of days on which the permissible air quality norms were exceeded during the heating seasons between 2013-2015 in Sosnowiec*

The concentration of airborne particulate matter in the city varies according to the type of atmospheric circulation (Knozová, 2012). The characteristics of the latter are often determined on the basis of the calendar of circulation types developed by Niedźwiedź, which distinguishes ten cyclonic situations, ten anticyclonic situations and one unidentified situation (Niedźwiedź, 1981).

In general, it can be concluded that higher concentrations are associated with anticyclonic situations (Leśniok et al., 2010, Bokwa, 2012). For the city of Sosnowiec, the mean concentration of PM10 particulate matter during anticyclonic conditions was

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55 µg/m^3, whereas the mean concentration of this pollutant in the case of cyclonic conditions was 45 µg/m^3 within the heating seasons of 2013-2015 (*Table 1*).

Table 1. *Mean, minimum and maximum concentrations of PM10 particulate matter in Sosnowiec depending on the type of circulation in the heating seasons of 2013-2015 (µg/m^3)*

No.	Type of situation	Mean concentration	Minimum concentration	Maximum concentration
1	Na	33	16	76
2	NEa	48	19	85
3	Ea	56	24	225
4	SEa	49	20	117
5	Sa	69	39	148
6	SWa	66	25	174
7	Wa	49	16	126
8	NWa	48	16	152
9	Ca	63	17	123
10	Ka	71	26	235
11	Nc	32	10	73
12	NEc	63	16	177
13	Ec	43	17	81
14	SEc	44	14	95
15	Sc	53	23	88
16	SWc	52	14	127
17	Wc	35	12	136
18	NWc	24	10	60
19	Cc	56	56	56
20	Bc	49	10	134
21	x	57	28	93
	$\bar{x}_a$	55	22	146
	$\bar{x}_c$	45	18	102
	$\bar{x}_x$	57	28	93

Explanations: N - north air advection; NE - north-east air advection; E- east air advection; SE - south-east air advection; S – south air advection; SW - south-west air advection; W – west air advection; NW – north-west air advection; Cc - central cyclonic conditions; Ca - central anticyclonic conditions; Ka – anticyclonic wedge; Bc – cyclonic trough; X - unidentified situation; A - anticyclonic conditions; C - cyclonic conditions

The highest concentrations of PM10 particulate matter in Sosnowiec during the heating season in the years 2013-2015 occurred during an anticyclonic wedge, on average amounting to 71 µg/m^3. The minimum concentration for this type of anticyclonic situation was 26 µg/m^3. The maximum concentration at the anticyclonic wedge was 235 µg/m^3, which is a significant exceedance of 200 µg/m^3. Above this level, there is a considerable risk to human health among particularly vulnerable groups of the population resulting from even a short-term exposure to pollution (Official Journal of Laws 2012 item 1031). In the case of anticyclonic advection from the south

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mean concentration was not much lower, at 69 μg/m^3. In this case, the minimum concentration was 39 μg/m^3, while the maximum was 148 μg/m^3. A high mean concentration also occurred in the anticyclonic south-west air advection, amounting to 66 μg/m^3. The minimum concentration for this type of situation was 25 μg/m^3. The maximum concentration was higher than that of the anticyclonic advection from the south, as it amounted to 174 μg/m^3. The occurrence of central anticyclonic conditions was associated with an average concentration of 63 μg/m^3, which is also significantly over the permissible limit. The minimum concentration was 17 μg/m^3, while the maximum reached 123 μg/m^3. In this case, the minimum concentration was lower than the permissible PM10 level in the air. Although the mean concentration for the anticyclonic advection of air from the east was 56 μg/m^3, which was significantly lower than the highest of the mean concentrations (71 μg/m^3), the maximum concentration was as high as 225 μg/m^3 reaching the highest level of all the situations. Anticyclonic conditions, such as north-east air advection, south-east air advection, west advection and north-west advection, shared very similar mean concentrations of 48-49 μg/m^3, not exceeding the permissible level of PM10 concentrations. The maximum concentrations were in the range of 85-152 μg/m^3. The lowest mean concentration of PM10 was noted during the anticyclonic advection of air from the north, at a level of 33 μg/m^3. In this case the minimum concentration was 16 μg/m^3, while the maximum was 76 μg/m^3.

In the case of cyclonic conditions, the highest mean concentration of PM10 particulate matter in Sosnowiec during the heating seasons in the years 2013-2015 occurred during cyclonic advection from the north-east and amounted to 63 μg/m^3. The minimum concentration was 16 μg/m^3, i.e. not exceeding the permissible level, while the maximum concentration was 177 μg/m^3. It should be noted that central cyclonic conditions occurred only once during the entire study period, so the relatively high concentration of 56 μg/m^3 for this type of circulation cannot be taken into account in the analysis. The high value could have been accidental, which cannot be determined without more data. Other than the central cyclonic situation, advection from the south was associated with the second highest mean PM10 concentration of 53 μg/m^3. The minimum concentration reached 23 μg/m^3 and therefore it did not exceed the permissible level. The maximum concentration was 88 μg/m^3. A slightly lower mean concentration of 52 μg/m^3 was associated with cyclonic south-west air advection. The minimum concentration did not exceed the permissible level and was 14 μg/m^3, while the maximum concentration was 127 μg/m^3. A mean concentration just below the permissible level of 49 μg/m^3 can be attributed to the cyclonic trough. It should be noted that the lowest of all the minimum concentrations of 10 μg/m^3 was noted for this type of situation, reaching the same level only with the cyclonic north-west air advection. The maximum concentration was 134 μg/m^3. Similar concentrations in the range of 43-44 μg/m^3 were associated with cyclonic advection of air from the east and south-east. The minimum concentrations were in the range of 14-17 μg/m^3, while the maximum concentrations were 81-95 μg/m^3. The mean concentrations during cyclonic advection from the north and cyclonic advection from the west ranged from 32 to 35 μg/m^3. Minimum concentrations were 10-12 μg/m^3, while maximum concentrations showed a greater variation, with values ranging between 73-136 μg/m^3. The lowest mean concentration in cyclonic conditions occurred with the north-west air advection, amounting to only 24 μg/m^3. This was at the same time the lowest average concentration among all types of cyclonic and anticyclonic conditions. The minimum concentration in this case was 10 μg/m^3, which, as mentioned above, is also the lowest

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minimum concentration occurring during a cyclonic trough. The maximum concentration was 60 µg/m^3 and was the lowest of all maxima, with the exception of the central cyclonic situation.

Unidentified situations were characterized by a mean PM10 concentration, remaining at a level of 57 µg/m^3. The minimum concentration did not exceed the permissible level, reaching a value of 28 µg/m^3. The highest recorded concentration for unidentified situations was 93 µg/m^3.

It can be observed that higher concentrations of PM10 particulate matter in Sosnowiec are associated with anticyclonic conditions. The worst air quality with respect to PM10 particulate matter throughout the heating seasons of the 2013-2015 period occurred during anticyclonic conditions. Also, higher concentrations of pollutants are correlated with air inflow from the south, irrespective of the anticyclonic or cyclonic nature of the conditions. The mean concentration for advection from the south is approximately 55 µg/m^3, compared to 41 µg/m^3 for north advection. The mean particulate matter concentration for east advection is 51 µg/m^3, while the mean concentration associated with advection from the west is 46 µg/m^3. The average concentration of PM10 for the north-east advection is 46 µg/m^3, while for the north-west advection it is 37 µg/m^3. The mean concentration of PM10 in the south-east air advection is 53 µg/m^3, and in the case of south-west advection, it is 54 µg/m^3.

Another important issue in addition to the concentration occurring for a particular type of atmospheric circulation is the frequency of this situation. During the period considered, the prevalence of anticyclonic events was 54.72%, while 43.24% of cases were cyclonic. Unidentified situations accounted for merely 2.04% of cases. During the days with exceeded permissible PM10 levels, the incidence rate was 66.51% for anticyclonic systems and 30.72% for cyclonic systems. The frequency of various atmospheric circulation types overall and during PM10 exceedances in the 2013-2015 heating seasons in Sosnowiec are presented in *Table 2* below.

Table 2. *Frequency of atmospheric circulation types during the occurrence of exceedances in the permissible levels of PM10 particulate matter in Sosnowiec during the 2013-2015heating seasons*

No	Air circulation type	Frequency %							
		2013		2014		2015		Average	
		Total	Exceedance	Total	Exceedance	Total	Exceedance	Total	Exceedance
1	*Na*	1.89	1.89	0.00	0.00	4.25	0.00	2.04	0.63
2	*NEa*	3.30	1.89	2.36	3.90	1.89	1.45	2.52	2.41
3	*Ea*	4.25	3.77	7.08	5.19	3.30	4.35	4.87	4.44
4	*SEa*	5.19	6.60	11.32	7.79	8.49	8.70	8.33	7.70
5	*Sa*	1.89	2.83	6.60	10.39	1.42	2.90	3.30	5.37
6	*SWa*	8.96	12.26	8.49	12.99	8.49	15.94	8.65	13.73
7	*Wa*	9.91	10.38	4.25	6.49	19.81	20.29	11.32	12.39
8	*NWa*	5.66	4.72	1.42	2.60	4.72	2.90	3.93	3.40
9	*Ca*	4.25	5.66	1.89	5.19	5.19	5.80	3.77	5.55
10	*Ka*	4.25	7.55	6.13	9.09	7.55	15.94	5.97	10.86
11	*Nc*	0.94	0.00	1.42	1.30	1.89	0.00	1.42	0.43
12	*NEc*	5.19	6.60	0.94	1.30	1.89	2.90	2.67	3.60

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No	Air circulation type	Frequency %							
		2013		2014		2015		Average	
		Total	Exceedance	Total	Exceedance	Total	Exceedance	Total	Exceedance
13	*Ec*	5.19	6.60	3.30	1.30	2.36	0.00	3.62	2.63
14	*SEc*	6.60	4.72	8.96	2.60	2.36	0.00	5.97	2.44
15	*Sc*	2.83	3.77	2.83	2.60	2.36	4.35	2.67	3.57
16	*SWc*	7.08	5.66	9.91	14.29	3.77	2.90	6.92	7.61
17	*Wc*	8.02	0.94	6.60	1.30	6.13	2.90	6.92	1.71
18	*NWc*	4.72	0.94	4.72	0.00	3.77	0.00	4.40	0.31
19	*Cc*	0.47	0.94	0.00	0.00	0.00	0.00	0.16	0.31
20	*Bc*	7.55	9.43	9.91	9.09	8.02	5.80	8.49	8.11
21	*x*	1.89	2.83	1.89	2.60	2.36	2.90	2.04	2.78
Σa		49.53	57.55	49.53	63.64	65.09	78.26	54.72	66.51
Σc		48.58	39.62	48.58	33.77	32.55	18.84	43.24	30.72
Σx		1.89	2.83	1.89	2.60	2.36	2.90	2.04	2.78

In the case of anticyclonic conditions, the most common type of atmospheric circulation in the heating 2013-2015 seasons in Sosnowiec was the west air advection, which averaged 11.32%, while during the period of exceeded permissible levels of PM10 particulate matter, the incidence of this type of situation amounted to 12.39%. It is worth noting that the anticyclonic advection from the west was the most common of all situations. The second most common in terms of the prevalence of anticyclonic conditions was the south-west advection, amounting to 8.65% of all situations. In the case of days with exceeded permissible PM10 levels, this situation reached a frequency of 13.75%. Despite the fact that anticyclonic advection from the south-west is less frequent than advection from the west, its appearance is more frequently associated with exceeded permissible levels of PM10 in Sosnowiec. The third most frequently occurring type of anticyclonic conditions was the advection of air from the south-east, occurring in 8.33% and 7.70% of cases during days with excessive particulate matter pollution levels. The anticyclonic wedge associated with the highest mean concentrations of PM10 occurred only in 5.97% of cases, but for days with PM10 levels above the permissible level, the frequency of the anticyclonic wedge was at a level of 10.86%. In spite of the moderate incidence of this situation, its occurrence is in many cases associated with the exceedance of permissible levels of particulate matter PM10. The lowest prevalence among anticyclonic conditions was observed in the case of north advection, occurring in 2.04% of cases. During days with PM10 levels exceeding the permissible limit, north advection was only 0.63%.

In cyclonic conditions, the cyclonic trough proved to dominate, at 8.49% and 8.11% on average observed during the days when PM10 particulate matter levels were exceeded in the city. The second most common type of cyclonic conditions was the advection of south-west air, which averaged 6.92% and 7.61% on days with exceeded permissible PM10 levels. Equally frequently, cyclonic advection of air from the west amounted to 6.92%, but during days with exceeded permissible levels of PM10 this situation occurred in only 1.71% of cases. Except for the central cyclonic situation, which occurred only once in the analysed period, the cyclonic north advection was least

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frequent at 1.42%, and only 0.43% on days when permissible levels were exceeded. It should be noted that cyclonic advection from the north was the least common type of atmospheric circulation.

Unidentified situations accounted for only 2.04% of the total, and 2.78% of the days when permissible levels of PM10 in Sosnowiec were exceeded.

Interesting conclusions can be drawn from the analysis of the mutual relationship between mean concentrations and the frequency of incidence. For example, a high mean concentration of 69 μg/m^3 is linked to anticyclonic advection from the south, but this situation occurs with a frequency of 3.30%, which is not high. It could be interesting to attempt to assign particular types of atmospheric circulation to groups of hazards (Ziernicka-Wojtaszek, 2012). Permissible levels of PM10 concentrations of 50 μg/m^3 and a median of the overall prevalence of 5.66% were chosen as parameters separating individual groups of hazards (*Fig. 4*).

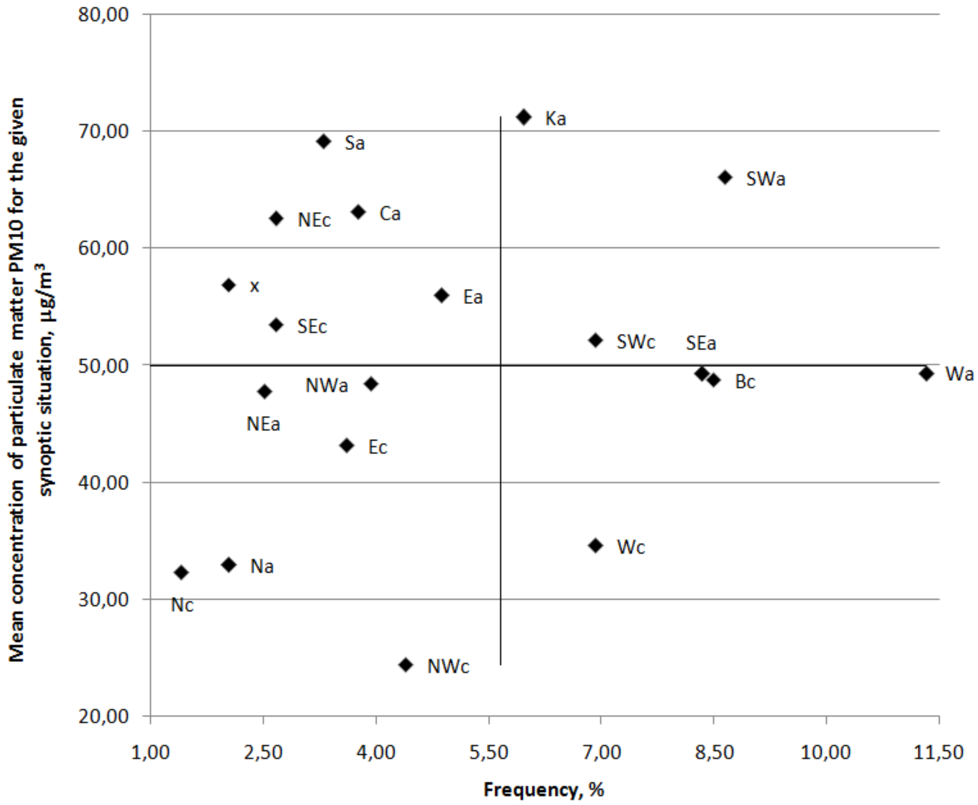

Figure 4. *Circulation conditions for the mean concentrations of PM10 in the 2013-2015 heating seasons in Sosnowiec*

These parameters can be divided into four groups. The first group, representing the greatest hazard, includes situations such as the anticyclonic wedge, the anticyclonic advection from the south-west and the cyclonic advection from the south-west. These situations are characterized by an average PM10 particulate matter concentration higher than the permissible level, and prevalence higher than 5.66%. The second group, representing a high level of hazard, concerns situations with a mean concentration of more than 50 μg/m^3 and a prevalence of less than 5.5%. This includes unidentified situations, anticyclonic advections from the south, anticyclonic advections from the

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east, central anticyclonic situation, cyclonic advection from the south-east, and cyclonic advection from the north-east. The third group, representing a minor hazard, includes situations such as cyclonic and anticyclonic advection from the south-east, anticyclonic advection from the west and cyclonic advection from the west. These situations are characterized by a mean concentration of PM10 below the permissible level, and a prevalence of over 5.66%. The above-mentioned situations comprise the fourth, low hazard group, characterized by an average concentration of PM10 below 50 μg/m^3 and the lowest frequency.

Discussion

This paper analyses the effect of the type of circulation on the PM10 particulate matter concentration in the air in the city of Sosnowiec. The impact of meteorological conditions such as air temperature, wind force, or precipitation values, which also affect airborne concentrations of pollutants, has not been analysed (Czarnecka and Nidzgorska-Lencewicz, 2008). Exceeded permissible levels of pollution with PM10 particulate matter in Sosnowiec pose a serious environmental hazard. Permissible levels of airborne particulate matter were exceeded pollution on about 40% of days in the heating seasons between 2013 and 2015. In this area anticyclonic conditions dominate, representing 54.72% of all days in the studied period. This result is confirmed by scientific studies addressing the issue of air quality (Demuzere et al., 2009), whose authors concluded that anticyclonic conditions predominate over Europe, and thus also in Poland. In cities, air quality is associated with anticyclonic conditions, which is consistent with the results of this research (Bielec-Bąkowska et al., 2011). The most unfavourable group of situations includes the anticyclonic wedge, the anticyclonic advection from the south-west, and the cyclonic advection from the south-west. The maximum concentration of PM10, at a level of 235 μg/m^3 occurred during the anticyclonic wedge. Anticyclonic advection from the north-west, anticyclonic advection from the north-east, anticyclonic advection from the north, cyclonic advection from the east, cyclonic advection from the north, and cyclonic advection from the north-west belong to the group associated with the lowest risk of PM10 exceeding the permissible level. Frequent exceedances of permissible levels are correlated with inflow of air from the south. A similar dependency occurs in other Polish cities (Leśniok and Caputa, 2009; Bokwa, 2012; Czarnecka and Nidzgorska-Lencewicz, 2015; Leśniok et al., 2010).

In the city of Sosnowiec and the Upper Silesia region, several studies have been conducted on the impact of synoptic conditions on PM10 concentrations from various periods in the past. Although the air quality in Sosnowiec and Upper Silesia is not satisfactory, there are positive trends in the reduction of pollutants due to excessive PM10 concentrations. Comparison of the results from 1994-2004 averaged from nine monitoring stations in Upper Silesia (Leśniok et al., 2010) with the results of this study for Sosnowiec from 2013-2015 shows that the mean PM10 concentrations are currently lower by 18 μg/m^3 for anticyclonic conditions and by 9 μg/m^3 for cyclonic conditions. For conditions where the concentrations are highest, i.e. SWa, the values are on average as much as 23 μg/m^3 lower. The downward trend of PM10 concentration in the air in the Katowice region is illustrated by the results presented in the graphs for the 1994-2004 period (Leśniok and Caputa, 2009). The positive trend of reduction of air pollution by PM10 in Sosnowiec is confirmed by the results for the number of days when permissible levels were exceeded in the 2013-2015 heating seasons in Sosnowiec,

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presented in this paper. These values in successive years were 106, 77 and 69 days. For 2009 the number was 108 days (Bielec-Bąkowska et al., 2011). It is also worth noting that in previous research of this type concerning the dependence of PM10 concentrations on synoptic conditions, only the mean concentrations for various synoptic conditions were given. What is new in the present study is the simultaneous presentation of both the mean PM10 concentration for each synoptic situation and the frequency of these situations. Despite the downward trend in particulate air pollution due to air protection programmes, the variability of PM10 concentrations in different synoptic conditions is very high. Conditions generating high and excessive concentrations are particularly interesting. The results of this study and future research may be used to predict high concentrations and exceedances of permissible PM10 levels on the basis of forecasts of synoptic conditions.

Conclusions

The following conclusions can be drawn from the research:

1. Daily PM10 concentrations in the heating season vary a great deal depending on frequently changing synoptic conditions. Higher PM10 concentrations are associated with anticyclonic conditions, during which the PM10 concentration was 55 $\mu g/m^3$, a compared to 45 $\mu g/m^3$ for cyclonic conditions. During the heating seasons in Sosnowiec, anticyclonic conditions predominate, accounting for 54.7%, and 66.5% on days when permissible PM10 levels were exceeded in the air.
2. The highest risk of exceeding the permissible levels of PM10 particulate matter in the city of Sosnowiec occurs in the anticyclonic wedge, but other hazardous situations include anticyclonic and cyclonic advection from the south-west. These situations are characterized by an average PM10concentration above the permissible level and a frequency of 5.66%.
3. There is a downward trend in the PM10 concentration both during the research period and in comparison to periods in the years preceding it, studied by other authors.
4. The relationships observed can be used to predict high concentrations of PM10 and exceedances of permissible levels on the basis of forecasts of synoptic conditions.

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COMPUTATIONAL FLUID DYNAMICS APPLICATION FOR THE EVALUATION OF A COMMUNITY ATRIUM OPEN SPACE DESIGN INTEGRATED WITH MICROCLIMATE ENVIRONMENT

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Abstract. This study aims to develop an approach which integrates the concept of climate-sensitive design with housing community design for the design of better residential buildings fitting into local wind environment. Employing research methods involving field investigation and computational fluid dynamics (CFD) simulation, the ventilation environment of four typical community atrium spaces were examined and compared in this study. Through a combination of CFD simulation and scenario-based design studies, the research results reveal that the typical long-shape building blocks with high-density housing development lead to poor natural ventilation in atrium open spaces. The research also indicates that if the openings of building masses and atrium can match the prevailing wind direction, the ventilation in community atrium open spaces will be significantly improved, but the overall effects are also affected by several design factors such as the distance between buildings, the size and shape of urban blocks, organization of building masses, and whether or not the wind corridor effect is effectively utilized. It is suggested for further research that more detailed issued-based CFD simulations (e.g., various land use controls or user preferences) of the relationship between wind environment and various design strategies should be conducted in the early stage of the entire design process to obtain the improvement in ventilation environments.
Keywords: *computation fluid dynamic (CFD); housing design; ventilation; wind environment; thermal environment*

Introduction

Given the impacts of global climate change and the urban heat island effect, sustainable design methods by which to create a community environment that better fits into the local microclimate characteristics has become an important research issue in both urban design and community design (Mirzaei and Haghighat, 2010; Jan et al., 2013; Hsieh et al., 2016; Yang et al., 2017). Climate change and the negative effects of urbanization on the living environment, such as high temperatures, increases in PM 2.5, and increases in the heat island effect, make the aerodynamic properties of urban areas increasingly important in architectural and urban design, especially in hot and high density urban areas (Chao and Ng, 2014; Yim et al., 2014). Wind passing from rural areas into a city provides cleaner and cooler air into urban canopy layers in summers and good ventilation has been noted as one of the possible mitigation solutions to improve urban air environments and mitigate the impact of heat island effect (Hang, 2013; Hsieh and Wu, 2012). Good ventilation not only helps eliminate pollutants, it also improves outdoor human comfort by accelerating heat exchange, as well as enhances the natural ventilation capacity of interior spaces (Givoni, 1998; Ishida et al., 2005; Xie et al., 2006).

Urban wind corridors can result from roads, open spaces, and passages through which air reaches the interiors of urbanized areas (Suder and Szymanowski, 2014). Over the years, urban wind research has investigated turbulent flow conditions in different street canyon models (Shi et al., 2015; Allegrini et al., 2015). Building blocks with limited open spaces between them, uniform building heights, and large podium structures have led to lower permeability for urban air ventilation at the pedestrian level (Ng, 2009; Yuan and Ng, 2012). High density housing development patterns and improper building layout in many dense urban areas have affected the ventilation of residential buildings as well as the comfort of residents (Walton et al., 2007; Du et al., 2017). These kinds of studies have shown that there are significant impacts of site planning and building layouts on the resulting wind environment around buildings. Therefore, it has become a great challenge for both urban designers and architects to determine how to better utilize the wind environment and natural ventilation to improve the outdoor wind environment as well as to enhance air quality in urban areas (Hsieh and Huang, 2016; Peng et al., 2017).

Previous studies have also revealed the importance of carrying out an environmental assessment of the available alternatives in the design stage of urban and building projects (Asfour, 2010). However, in spite of the fact that some research effort has been made, the lack of good interaction between urban wind environment researchers and urban space designers, together with a shortage of easy-to-use and easy-to-understand simulation tools for evaluating design alternatives in the design process has limited the practical application of related research. In terms of methodology and analytical tools, the effects of the urban building arrangement on the wind environment have been investigated through wind tunnel experiments or numerical simulations, such as Computational Fluid Dynamics (CFD). CFD has become a commonly used numerical simulation method and analytical tool for studying the wind environment (Takahashi et al., 2004; Mochida and Lun, 2008; Hu and Ryuichiro, 2013). It has some important advantages over wind tunnel testing. For example, wind tunnel measurements are generally performed at a few selected points under similar requirements in urban models. CFD, on the other hand, is conducted at full scale and provides whole-flow field data and information on the relevant parameters at all points of the computational domain (Blocken et al., 2012; Mirzaei and Caemeliet, 2013) Moreover, CFD is being increasingly used to assess pedestrian-level wind conditions in urban areas (Wu et al., 2013). Because of these advantages, CFD is being increasingly used to study a wide range of wind-related environmental problems in urban areas, such as urban air pollution and natural ventilation of community building design (Gromke et al., 2015; Mora-Pérez et al., 2015; Wang and Li, 2016).

It is also worth mentioning that, thanks to the development of numerical simulations, a more efficient design approach and design project evaluation has been made possible. Generally speaking, the entire design process normally includes three stages: a pre-design stage, a design stage, and various post-design stages. In an age when digital simulation tools have not been widely applied in design practice, many conceptual design ideas have been difficult to put into practice because they could not be validated before being mass produced. Also, in a design age that is heavily reliant on drawing tools such as pens and papers, it is quite often the case that many design products have

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been manufactured without pre-design evaluations. Although sometimes post-design evaluations or post occupancy evaluations (POEs) have been conducted to help identify some design mistakes, it is often too late to make any adjustment because the products or buildings have already been manufactured.

Similar cases in community housing design, systematic considerations of the topic are limited and normally not included in decision-making at the pre-design and design stage in community planning and design. In spite of these effects being noticed by researchers, relatively few efforts have been made by urban designers and architects to integrate the effects of natural ventilation into block-level community housing design. In high density Asian cities for example, in order to allocate the maximum number of housing units or maximum Floor Area Ratio (FAR), natural ventilation is normally not seen as a key consideration in organizing housing layout and building masses. Moreover, local building codes in many Asian cities also have failed to take the effect of the wind corridor into consideration in arranging building setback and design guidelines for community open spaces.

Unlike the past approach, current applications of digital simulation tools such as CFD, enable design problems to be identified in the pre-design stage or sketch design stage. Moreover, with the help of computer simulations, an integrated product evaluation process that links the design stages, simulations, and assessments can be established before products are actually manufactured. For example, the development of CFD has resulted in the widespread use of this technique not only as an environmental research tool but also as an architectural design tool. In view of this dilemma, this research is an attempt to address the issues indicated above by using four prototype building layouts in Tainan City, Taiwan as case settings. The practical applications of wind engineering (CFD modeling) on architectural design and community open space design are discussed to together with considerations of housing market requirement. This study is aimed toward changing traditional experience-based design methods toward more scientific wind environmentally-sensitive architectural design an community design, in order to ensure that the surrounding wind environment of the proposed residential buildings is adequately considered. Employing research methods involving field investigation and CFD simulation, this study is an attempt to explore two research issues: (1) how to systematically analyze the influence of community building layouts and atrium designs on the ventilation of community outdoor spaces and (2) how to suggest suitable building layouts and open space design principles that strengthen the coordination between the local wind environment and the design of community open spaces. Based on our empirical approach, the effect of building arrangements on average ventilation efficiency is numerically evaluated in selected residential areas at the pedestrian level, and practical design suggestions are proposed to serve as a guide for better urban ventilation design.

Materials and methods

Study area

Tainan City (23°0’16’’N, 120°13’10’’E) is located in a tropical climate zone, considerably close to the Tropic of Cancer which divides the subtropical zone and tropical zone. Known as the fourth most populated city in Taiwan, Tainan City is also highly urbanized. The heat island intensity, defined as the maximum air temperature

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difference between the urban and rural areas of Tainan City during the nighttime and daytime, is 3.4 °C and 3.2 °C, respectively (Lin et al., 2005; Lu, 2008).

According to data from years 2001-2016, the average temperature from June to September was more than 28.5 °C. Among them, the temperature in July was the highest, recorded as 29.6 °C during the summer season (Hsieh et al., 2016). Furthermore, Tainan City is close to the sea and therefore having high humidity in summer. The wind environmentally-sensitive architectural design which consider how the natural ventilation can be utilized becomes an crucial step to improve the outdoor wind environment. The above weather characteristics, make Tainan City a good candidate for wind environment analysis, one of the main objectives of this study. It is a common issue in the southern part of China and also the Southeast Asia areas, especially for those places with the similar weather characteristics of high air temperature and humid in summer (Feriadi and Wong, 2004; Nguyen and Reiter, 2014; Priyadarsini et al., 2014; Sharmin et al., 2017).

Field measurement design

To achieve the purposes of this research, a representative residential neighborhood next to Barclay Park in Tainan City, Taiwan (a subtropical climate zone) was selected as the empirical case setting. The residential neighborhood includes four types of housing communities with different building masses and building layouts. Each of them is a complete block development, and the buildings are typical housing types in Taiwan. Given that there are four prototypes of residential building layouts appearing in the same microclimate environment, this residential neighborhood provides an excellent case for addressing the relationship between the outdoor wind environment and building layouts as well as the effects of atrium design. The location and current situation of study area is shown in *Fig. 1*.

Figure 1. *Location map of study area*

The famous Barclay Ecological Park is located on the east side of the study area, and a large agricultural experimental farm was located on the south, which has been just rezoned by the Tainan City Planning Commission as a new urban development site, but new land development has not occurred yet. To the north is a 40-M main road, and to the west are a large parking lot and some low-rise houses. Given that the prevailing wind direction in summer of the study region is mostly southerly, it can be assumed that the impact of the surrounding buildings on the local wind environment is small.

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The selected residential neighborhood (the study area) contains four different types of atrium housing communities (*Figs. 2* and *3*). Type A Community contains one 63 m high-rise elevator building complex (three sets of connected 20-story apartment buildings), which has a small-scale unilateral open atrium facing the south. Type B Community contains two blocks; each has several connected 7-story apartment buildings, with a height of 24 m and a narrowed atrium with two unilateral openings. Type C Community is a townhouse residential community shaped by 4 unit building complexes with a building height of 16 m. It contains two residential blocks; each has a ribbon-shaped atrium open space with three openings. Type D Community is a row house residential community shaped by connected 5-story buildings (16 m), with the opposite side opening into a ribbon-shaped atrium in each block. All the blocks are densely developed in order to obtain the maximum number of housing units, and this is a common situation in land development practice in Taiwan in order to increase economic profit.

Figure 2. *Open spaces of residential community atrium in study area.(a) Community: elevator apartment with opening on one side. (b) Community: 7 stories apartment with two openings on one sides. (c) Community: belt atrium community with opening on two sides. (d) Community: belt atrium community with opening on the edge*

Figure 3. *Measurement equipment*

The measurement time of our survey was between July 5th and July 28th of 2014. This three weeks period in Taiwan is when the weather is the hottest, and the impact of the urban heat island is the strongest. Therefore, a study on how to enhance the ventilation of atriums in such communities is critical in order to create a comfortable environment for outdoor activities. The microclimate measurement was conducted on

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selected spots, at selected time periods in the mornings and afternoons during the three weeks (9:00-11:00 in the morning and 2:30-4:30 in the afternoon) when the weather conditions were typically torrid without obvious strong wind. The major factors examined in the survey were wind speed, temperature, humidity, and the perceived comfort of users of each atrium space. The measurement equipment included Cambridge Accusense UAS 1500 anemometers (*Fig. 3*), AM-4214SD anemographs, and HT3007SD hydro-thermal meters. The Cambridge Accusense UAS 1500 anemometer, whose accuracy can reach 0.10~20 m/s ± 5 %, was assembled on a tripod 1.5 m above the ground (the range of wind often affects pedestrian comfort). With regard to the arrangement of the measurement points (*Fig. 4*), the points were carefully selected to reflect the critical points that influencing atrium wind fields and user comfort, including points in the main nodes of outside activity spots and wind paths, and in the opening of the atriums. Effort was also made to keep the survey points equally and spatially distributed. Because the Type A Community is the highest building in the study area, a measurement point was set on the floor of the attic to measure the surrounding wind speed, which was imported into the CFD analysis later as a baseline parameter.

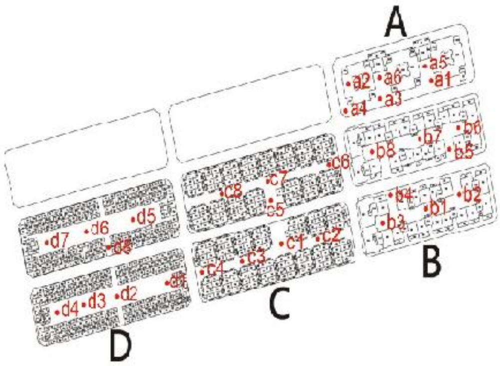

Figure 4. *Map of measurement points*

CFD simulation

Computation fluid dynamics (CFD) numerical simulations were employed to analyze the surrounding wind environment of the buildings. In regard to the setting of boundary conditions for the CFD simulation, the velocity inlet was set to be the gradient wind, where the vertical wind speed distribution was represented by the power law profile (*Eq. 1*). The land surface was set to be a no-slip condition.

$$\frac{U_{(z)}}{U_0} = \left(\frac{z}{\delta}\right)^{\alpha} \qquad \text{(Eq.1)}$$

where

U_0: wind speed (m/s) outside of boundary layer (also known as the gradient velocity)

$U_{(Z)}$: the wind speed at the height of Z(m/s)

Z: the reference height (m) (set to be the height of Building A = 60 m in this case)

δ: the height of gradient (m)

α: power law value (index)

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In accordance with relevant research practices, the height and the power law value of the boundary layer was determined depending on ground conditions. The simulation area was dominated by middle-story buildings, so the power law value was set at 0.25, and the gradient height was set at 400 m. The remaining parameter settings are shown in *Table 1*.

Table 1. *Parameters of boundary condition settings*

Items	Setting
Wind direction	S
Wind velocity (m/s)	3.40
CFD scheme	Central difference scheme
Governing equations	Navier-Stokes equations
Boundary volume (X×Y×Z)	1500 m(N-S)×1500 m(E-W)×450 m
Total mesh number	7,957,000 meshes
Reference height (δ)	60 m
Power law value (α)	0.25

In the case under study, the simulation area is surrounded mainly by a park, a large area of agricultural land and a large parking lot. Because there are no buildings of significant mass in the major wind directions of the study area, the influence of surrounding buildings on the wind environment was considered to be negligible in the model calibration. When calculating the flow field using a CFD model simulation, the setting up of a reasonable grid system and selection of suitable boundary conditions are key requirements for efficient computer calculation. Based on the experiences discussed in previous relevant studies, the boundary condition for the simulation was set to be 10 times of the height of the main buildings, and the height of the calculation area was set to be 5 times of the height of the main buildings. A structural grid was used in order to enhance the simulation accuracy. Due to the fact that the fluid variation around the buildings is much larger than that around other areas, a more refined grid system was developed around the buildings in order to get more accurate simulation results. WindPerfectDX was the software employed in this research. Its advantages include the capacity to develop a very fine grid system for a simulation as well as the ability to import the Sketch up 3D files easily and directly. Also, it has a strong visualization capacity for simulation results, which makes communication with developers and architects much easier.

Results

In order to validate whether the simulation results could be used for reference to community planning and design, a simulation model based on the current situation was first calibrated, and then the CFD simulation results were compared with the results of our microclimate survey conducted on the site. The model validation process involves making sure the CFD simulation model is reasonable and that the results are accurate and could be used for the evaluation of design plan. After the model validation process,

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the proposed alternative plans were then analyzed and compared. The model validation was conducted using the measured wind speed and prevailing wind direction, which is southerly. The wind speed measured at point a2 on the roof of the Type A 20-story building was used as the initial inflow wind speed.

The simulation results were compared with the actual measured results for the measurement points to examine the accuracy of the simulation model. Part of the results are shown in *Fig. 5*, which is a simulation based on southerly wind at an average speed of 1.3 m/s. The results indicate that the simulation values at points a6, b1, b5, c1, c8, d5 were all very close to the measured values, indicating that the model was reasonable. The simulation results of measurement points a1 and d2 were slightly different from the measured values, but the differences were both within an acceptable level (less than 0.09 m/s). In addition, a correlation analysis between the simulation values and measured values was conducted for all 30 measurement points, and the results showed a correlation coefficient r = 0.86, which indicates that the simulation results were close to the measured results. This further confirmed that the CFD simulation model could be used in the subsequent scheme assessment and analysis.

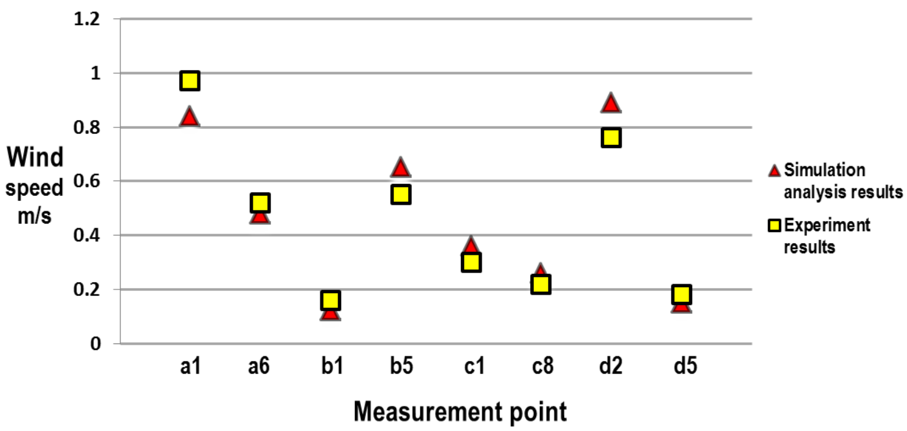

Figure 5. *Measurement point comparison between simulation analysis results and experiment results*

Evaluation of simulation results for community development plan on different blocks

After confirming the reliability of the proposed simulation model, a CFD simulation was conducted to evaluate the atrium ventilation performance of the four types of housing communities. The main process of the analysis involves calculating the spatial distribution of wind speed at pedestrian height in the outdoor space of the four communities. The CFD simulation was conducted using the prevailing wind direction in the summer. According to a field survey and the information obtained from a local weather station, southerly wind was used as the prevailing wind direction. The simulation results are shown in *Fig. 6*. The results reveal that “the position in which buildings face the wind field” and “the form and position of the openings in the community atrium in relation to the wind field” are two key factors influencing the ventilation condition in the community atrium situation. For the assessment of human comfort from viewpoint of landscape architects and urban planners, the high wind speed ($u \geq 1.0$ m/s) and low wind speed ($u < 1.0$ m/s) areas were defined (Hsieh et al., 2016). Moreover, pedestrian-level natural ventilation in the street canyon was assigned as the

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stagnant (u < 0.3 m/s), poor (0.3 m/s ≤ u < 0.6 m/s), low (0.6 m/s ≤ u < 1.0 m/s), satisfactory (1.0 m/s ≤ u < 1.3 m/s), and good (u ≥ 1.3 m/s) in Hong Kong (Yuan and Ng, 2012). According to previous studies and the results of our interviews with community residents, a wind speed of pedestrian field between 1.0 m/s and 2.5 m/s is a more comfort wind range for community atrium open space design, and the atrium open spaces of the four selected housing communities in urban blocks were examined subsequently.

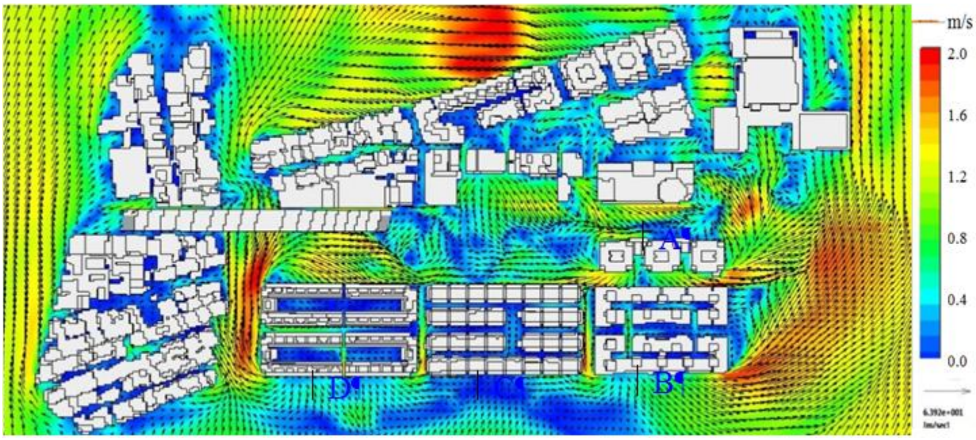

Figure 6. *CFD results of residential community environment (regional scale)*

The atriums, directly facing the direction of the summer prevailing wind, should have a better ventilation conditions compared to those of housing community type A. However, our analysis results show that the type A residential buildings in the back of building blocks perform better in terms of outdoor atrium ventilation. A possible explanation for this phenomenon is that the building blocks facing the windward side (building blocks on the outside of south facade) in type B and type C housing communities did not provide a corresponding opening on the windward side, so the airflow has to get in from the side entrances and the atrium entrance on the back side of the buildings, which leads to poor atrium ventilation. As for housing community type D, although these have openings on the windward side, the size of the opining is relatively small, and the atrium is too narrow and long, so the wind corridor effect can not expand to the entire atrium, which therefore leads to poor ventilation of the entire outside open space.

The research results also reveal that if there are sufficient openings provided in linear atrium communities (housing community type C), or if the position of the atrium opening is helpful for enhancing the airflow convection of the atrium (housing community type B), the outside ventilation condition will be significantly improved in areas that can be affected by air convection. In addition, our empirical results also indicate that “the relationship between the axial building layout and the wind corridor” and “the separation distance between buildings” both have obvious influences on community outdoor ventilation. For example, the arrangement of the long axis in housing community type C caused failure to match the prevailing wind direction and thus contributed to a weak effect of the wind guiding function. Similarly, housing community type D has only a narrow opening facing the windward side, which also limits the wind guiding effect of the passing wind corridor.

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Discussions

After exploring the possible reasons leading to poor atrium ventilation in the study cases, two alternative community design plans were proposed, and a CFD simulation was used to evaluate the effects of each plan. By considering consumer demand, housing development and local housing market trends (the demand for maximization of total floor area and the total number of housing units in order to make significant profit) as well as the principles of community ventilation design, two refined alternative plans were provided: one is a minor adjustment plan, and the other is a complete block redesign plan. Together with the plan of "business as usual" (the current one), there were a total of three plans to be simulated and compared. The architectural perspectives and selected elevation views of these plans are shown in *Figs. 7* to *9*.

The two alternative plans have different planning goals and considerations. The minor adjustment plan (community residential building plan 2, *Fig. 8*) adds ventilation openings and vents in selected buildings, while trying not to reduce the total floor area and total housing units in order to meet the expectations of developers who are seeking profit.


(a)
(b)

Figure 7. *Community residential building plan 1 (current situation). (a) Perspective view. (b) The south elevation view*

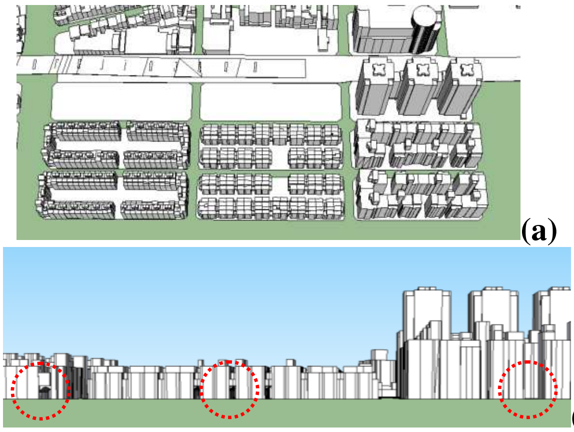
(a)
(b)

Figure 8. *Community residential building plan 2 (slightly-adjusted situation). (a) Perspective view. (b) The south elevation view*

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(a)

(b)

Figure 9. *Community residential building plan 3 (climate-sensitive adjustment situation). (a) Perspective view. (b) The south elevation view*

The design methods of the plan included adding one opening on the south side of the buildings of Community Type B, and then transferring the decreased floor area resulting from the new opening to the top floor of the buildings on both sides of the community entrance. This creates double effects: it maintains the total floor area on the one hand, while creating an enhanced image of the community entrance. A similar approach was used in Community Type C, where several air vents were added to the three connected building units in the middle building complex facing south, while the decreased floor area was added to the top floor of the buildings near the community entrance to create enhanced visual effects. As for Community Type D on the left, air vents were placed at the junctions of the building units on the four corners, and the decreased floor area was added to the top floor of the buildings next to the community entrance to enhance the entrance image.

The above-mentioned minor adjustments were made on the basis of a planning goal to add ventilation openings and vents to existing building forms and building masses, while trying not to decrease the total floor area and the number of housing units. Based on our interviews with the local land developers, this approach was highly accepted by them. The actual effect of the improvement atrium ventilation in this approach, however, will need to be examined based on the results of the simulation analysis.

As for the second alternative plan (community residential building plan 3, *Fig. 9*), it involves a large adjustment in which building blocks and building type mix are redesigned based on the concept of climate-sensitive design and the consideration that without significantly reducing the allowed floor area ratio of land development. The original two long narrow shaped blocks in Community Type B, Community Type C and Community Type D are combined into one block for each community. The size of the new block is about 80 m ×100 m, which is a block size good for human walkability and a better arrangement for building layout. The building types and arrangement of the building masses are also redesigned, and the scale of atriums, the building configuration, and the distances between buildings are all reconsidered so as to try to obtain the optimal atrium ventilation. Moreover, what is worth mentioning is that a combination of town houses and mid-rise apartment buildings are adopted in the building forms of Community Type C and Community Type D. The combination of different housing types and sizes reflects different housing prices and market demand,

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which is helpful to introduce households with various socio-economic backgrounds, and therefore contributes to the diversity of the population in these communities.

The simulation results for the three plans using CFD software are shown in *Fig. 10*. *Fig. 10* shows an enlarged image of the four communities. These results reveal that the two refined schemes, to various degrees, enhanced the natural ventilation of the atrium open spaces.

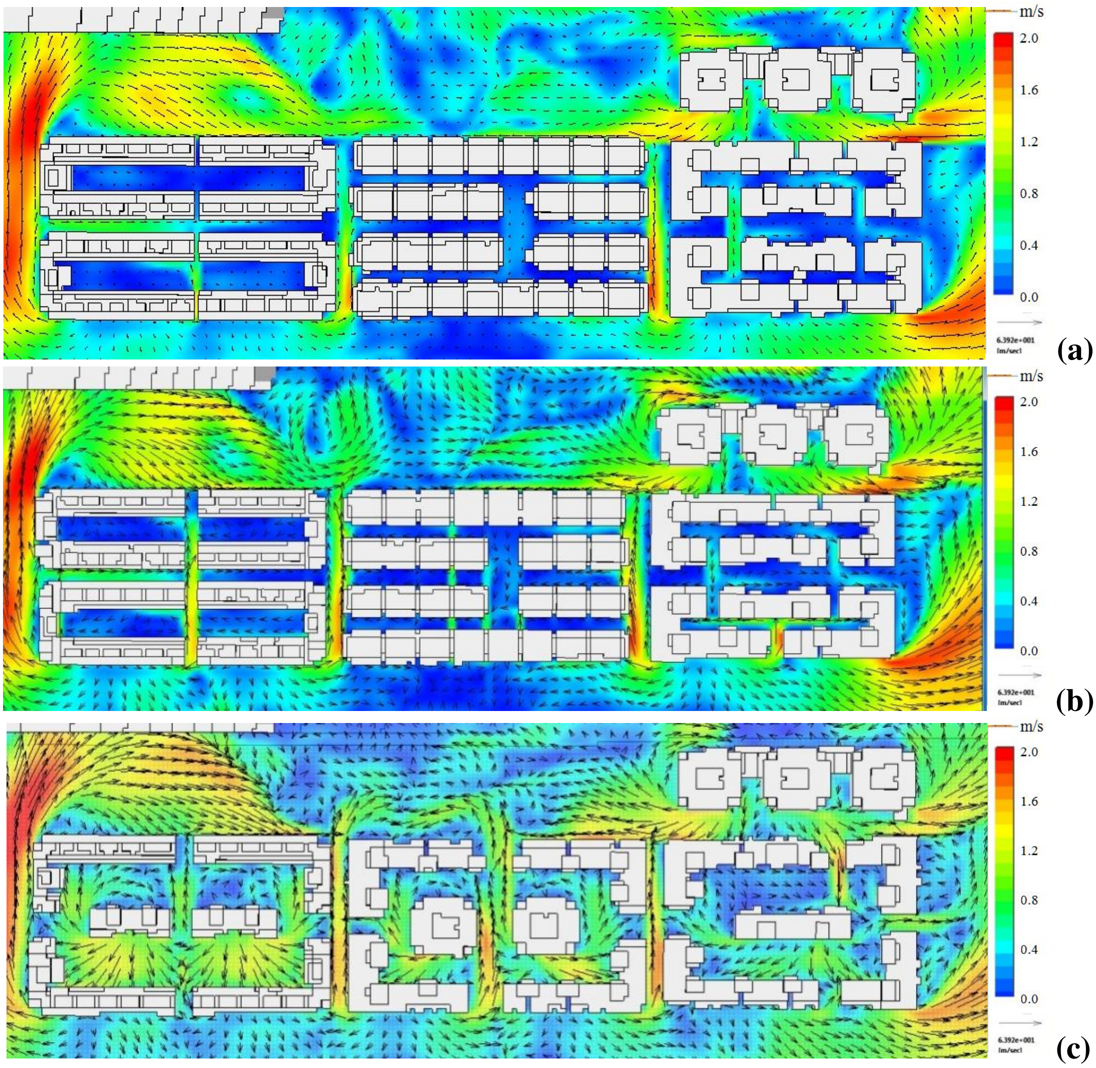

Figure 10. *The CFD analysis result comparisons among three community building setting plans (partially enlarged drawing) (a) Current Plan. (b) Slightly-adjusted plan. (c) Climate-sensitive adjustment plan*

For the minor adjustment plan (Plan 2), improvement of outdoor natural ventilation can be found in the areas close to the added ventilation openings or vents, such as those areas in housing Type B. However, for the community Type C, because the distance between the buildings is too short, and the linear atrium is too narrow and long, the effect of the three newly added ventilation vents was found to be very limited, and the effect of wind guiding and natural ventilation resulting from the vents was not able to expand to the full scope of the atrium. Similar situation occurred in Community Type

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D, where the newly added ventilation vents in the four corners partially improved the natural ventilation of the nearby areas on the windward side. However, given that the community has a very closed narrow-shape atrium with only two small openings to the outside wind corridor, the effect of the newly added vents on the improvement of natural ventilation in the atrium were also found to be very limited.

In terms of the complete adjustment plan (climate-sensitive adjustment plan) (Plan 3), which redesigns the building blocks, housing types, building layout, and atrium openings based on the characteristics of the local wind corridor and the design principles of outdoor natural ventilation, the results of the CFD simulation met our expectations. As indicated in *Fig. 10*, for all the Housing Types B, C, and D communities, the CFD simulation results the natural ventilation of their atrium open space to be significantly improved, both for the entire atrium and for partial areas in the atrium. For the case of climate-sensitive adjustment plans, it was showed from the simulation results that the wind speed areas in the stagnant and poor wind speed classes were largely decreased. This indicates that in order to obtain the optimal effect of natural ventilation for a community open space, it requires a comprehensive consideration of the size of the building block, organization of building types, building masses, building layout, and the community atrium openings. Also, a careful study of the relationships between these design factors and the local wind corridor is indispensable.

Similar with the climate characteristics of high hot and humid in South-East Asia, this study area also has similar climate, and therefore the design issue of ventilation are needed to be considered. In Vietnam, natural ventilation was found a better choice for passive cooling due to its hot and humid climate (Nguyen and Reiter, 2014). In Jogjakarta, people seemed to prefer higher wind speed and showed a tendency of creating higher air movement to modify the hot and humid living environment (Feriadi and Wong, 2004).

Recent studies have shown that the geometry and aspect ratio of urban canyons play a crucial role in moderating the microclimate at the street level (Xie et al., 2006; Asfour, 2010; Du et al., 2017). Heights and layouts of building group were found to influence ventilation, and step up configuration can distribute the wind evenly and allow the wind to reach the leeward side of each building. It was shown very effective in improving the overall natural ventilation and thermal comfort at pedestrian level (Rajagopalan et al., 2014). Another research in Dhaka used H/W ratios as an index originally to represent uniform and diverse geometric character of different sites (Sharmin et al., 2017). In our study, we also found the building arrangement, building layout and atrium openings were important design issues for a good ventilation. However, only few openings facing the windward side was found limited for ventilation improvement. The different height of buildings in an urban block such as the combination of town houses and mid-rise buildings shown in this study were one of the better design criterion to create a better wind environment.

Besides hot and humid environmental analysis considered in this study, the other aspects that most of the above papers were not discussed is that we took land development, urban planning and residential type preferences into consideration. It was shown even under the premise of housing preferences and economic profits of land development, it is still possible to enhance ventilation environment of the external space of communities. All the above findings can be applied to other tropical cities.

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Conclusion

In order to provide a useful methodology and a suitable operational tool to integrate housing community design with climate-sensitive urban design, this study demonstrated a research approach which improves the natural ventilation of community atrium open spaces on an urban block scale with the help of a CFD simulation. Based on a review of related theories and literature as well as the analyses of current situation and suggested design alternatives, the study results show that this methodology has practical value in assisting community housing planning and design and is particularly useful for developing draft evaluations of strategic planning and design in determining building layouts and masses on site planning, where the simulation outcomes from this method could be used to modify design concepts and strategies. Moreover, by utilizing the proposed CFD simulation to analyze suggested scenario-based design alternatives to the natural ventilation of blocks and residential buildings as well as comparing the simulation results of the proposed improvements, this research shows that the method may enhance the scientific base of the traditional urban design approach because it can diagnose planning and design problems in the early stage of the design process and therefore guide the modification of design schemes on a timely and cost-efficient way.

Referring to practical community planning and design, the study analyzed and compared four typical types of housing community design and their atrium ventilation conditions. After a review of relevant literature, the CFD simulation parameters in our modeling approach were verified through on-site field survey of the microclimate situation. Subsequently, the atrium ventilation performance of four different housing communities in the selected four adjacent blocks was examined and compared through CFD simulations. This provides an operational method to integrate the consideration of climate-sensitive design approach into community design process.

Under the simulation condition with a prevailing wind from the south (long axis of the buildings facing prevailing wind), the research results indicated that the typical long-shape urban blocks with high density in southern Taiwan have poor natural ventilation in their atrium open spaces. The research also shows that if the openings of building masses and atrium could be arranged according to the prevailing wind direction, the community atrium ventilation could be improved greatly. It was also found from a comparison of the results of various design schemes that the natural ventilation in community atrium open spaces can be significantly optimized by properly controlling design factors including: the size and shape of urban blocks, the distances between buildings, organization of building masses, and the coordination of the openings of buildings and atrium open space with the direction of wind corridors. Furthermore, the results show that if a consideration of wind environment can be conducted in the early stage of community planning and design process, together with a proper CFD wind simulation approach, improvement in community external ventilation environments can be obtained without significantly reducing the expectation of developers who often attempt to meet the requirements of taking full use of all allowed Floor Area Ratio (FAR) and build more housing units to generate economic benefits in land development. In fact, this study reminds us that a good ventilation environment in community atrium open spaces will lead to a comfortable residential environment. If this concept can be accepted by developers and home buyers as well as be capitalized into property values, and subsequently lead to some adjustments in housing design preferences which take the concept of wind-sensitive design into consideration, more

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and more residential communities will be built with good ventilation environments, and therefore the goal of climate-sensitive urban design will be realized sooner than later.

Finally, it is worth mentioning that this paper provides a methodology and operational approach to integrate the concept of wind-sensitive design with community outdoor space design within the requirement local housing market practice and the expectation of land developers. But there are still many issues and limitations needed to be addressed in local housing market environment as well as in zoning, urban design, and building code regulations. It is suggested that more detailed and issued-based simulations of the relationship between wind environment and various design strategies and alternatives which meet local context and content should be conducted before setting up real design guidelines in conducting urban design control.

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CO_2 EMISSIONS IN IRAN FOR 1990–2010: A DECOMPOSITION ANALYSIS

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Abstract. For over a decade now, decomposition analysis has become popular in energy and environment studies. This paper therefore identifies the factors that have influenced changes in CO_2 emissions in Iran for the period 1990–2010. The Refined Laspeyres Index method has been utilized in order to decompose the CO_2 emissions while four main factors of the observed changes have been identified: economic activity, energy intensity (energy use per unit of economic output), carbon intensity (CO_2 emission per unit of energy use), and population effect. The study period has been divided into two decades, the first one encompassing the period from 1990 to 2000 and the second one from 2000 to 2010. Our empirical findings have revealed that the main factor which is accelerating CO_2 emissions in Iran is the economic activity for the entire study period. Also within the first decade of the study, economic activity has been followed by energy intensity while in the second decade; it has been followed by population effect. Furthermore, the carbon intensity has made minor contributions to CO_2 emissions in Iran throughout the study period. Given that Iran is an energy intensive and largely oil dependent country; we therefore suggest it would be better off in terms of reduced CO_2 emissions by pushing down current energy intensity as its main environmental and economic policy objective. For the future studies, a sectoral CO_2 emissions analysis could be conducted for Iran in order to examine the carbon emissions increasing and decreasing factors and provide some sectoral policy implications.
Keywords: *environment, energy, sustainability, pollution, intensity, carbon, population*

Introduction

Environmental problems, especially climate change and global warming, have been on the world's agenda over the past several decades (Halder et al., 2013; Kuo and Perng, 2016; Lin, 2017). These problems have also raised the concern of energy analysts and policymakers about environmental sustainability. Many scientists argue that the increasing amount of CO_2 concentration in the atmosphere may create the greenhouse effect and this may cause global warming (IPCC, 2007). As Lotfalipour et al. (2010) states the main source of increasing CO_2 emissions in the atmosphere is fossil fuel combustion. The main factors that are raising fossil fuel consumption can be linked to economic development, technological changes, demographic changes, institutional frameworks, lifestyle, and international trade (Hatzigeorgiou et al., 2008).

Most of the Asian countries achieved a spectacular economic growth performance in recent decades (Zhang, 2008). As the Energy Information Administration (EIA) states, amongst these countries Iran is an energy superpower since it accounts for the world's second largest natural gas reserves and fourth largest oil reserves in 2015. Ninety eight % of Iran's overall energy demand is generated from natural gas and oil. On the other hand, Iran has the 7th rank in World Bank's CO_2 emissions ranking. Besides its one dimensional structure (depends mostly on oil revenues) Iran's economy expanded by

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139 % in real terms between 1990 and 2010 while the country's CO_2 emissions increased by 170.7 % in the same period (World Bank, 2015). Since the acceleration in Iran's CO_2 emissions is faster than its GDP increase this could be evidence that Iran's economic development is not environmentally sustainable. For instance, in China (which has experienced the most spectacular economic growth in Asia) the real GDP increased by 630 % while its CO_2 emissions increased by 236 % between 1990 and 2010 (World Bank, 2015).

In recent decades, there is an ongoing debate about the environmental impacts of high energy consumption among countries (Kumbaraoglu, 2011). Parallel to this, social and environmental aspects of energy sector projects gained importance in Iran. Accordingly, it is possible to state that there is an increasing awareness about energy production, conversion and utilization in Iranian government, industry and public.

In Iran electricity and heat production are the major activities which create CO_2 emissions. They constitute 32 % of overall Iranian emissions in 2010 (World Bank, 2015). In Iran energy prices are low and winter months are cold, therefore electricity consumption is increasing during the winter. Since 95 % of electricity supply is generated using oil and natural gas then this yield to an increase in CO_2 emissions (World Bank, 2015). The second major CO_2 emitting activities are manufacturing and construction. As the World Bank data indicates 25.8 % of overall CO_2 emissions arise from manufacturing and construction. As Moshiri (2012) states, Iran's industrial sector uses old technologies which are inefficient in terms of energy conversion. Transportation is another sector which increases Iran's CO_2 emissions. Transportation technologies are old in Iran and there exist high subsidies on gasoline (Moshiri, 2012). Therefore CO_2 emissions due to the transportation are increasing. Residential buildings and commercial & public services are also contributing to Iranian CO_2 emissions. This is probably due to the insulation deficiencies of the residential and commercial buildings and the use of high energy consuming home appliances. To sum up, it is possible to state that in Iran almost the whole of the economic activities are energy intensive and such high energy intensity increases the CO_2 emissions.

Together with the increasing awareness about CO_2 emissions reduction among the world countries, analyzing and understanding the factors that cause emissions increase has gained importance. Iran's CO_2 emissions exceeded 571 thousand KT in 2010 and this value is higher than those of some other countries that have similar GDP and population. Turkey can be given as an example to these countries. For instance Iran's CO_2 emissions are 1.9 times greater than Turkey's overall CO_2 emissions, where Turkey has almost the same population and higher GDP as compared with Iran (World Bank, 2015). Accordingly, analyzing the factors that accelerate or decelerate CO_2 emissions in Iran has gained great importance over the years. Identifying the components that are changing the CO_2 emissions is an important input to the policy makers and energy analysts who are developing some economically and environmentally sustainable projects. In addition, due to the geographic location Iran has the potential to diversify its energy sources. The identification of the sources of CO_2 emissions in Iran has not been widely studied. Published studies are mostly for developed countries and some developing countries, such as, China, India, Turkey, and Korea.

What are the factors that change the CO_2 emissions? This question can be answered by identifying the most important factors contributing to the overall changes in CO_2 emissions. One possible way for analyzing the CO_2emission changing factors is through econometric regressions. For instance, in his work Halicioglu (2009) proved that there

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exist a long run relationship between CO_2 emissions and income, energy consumption and foreign trade. Another possible way for analyzing the factors changing CO_2 emissions is the decomposition analysis (Kumbaroğlu, 2011). The method adopted in this paper is the decomposition analysis. The paper contributes to the literature by decomposing the CO_2 emissions in Iran, in a detailed way.

The aim of this study is to identify the factors that have been changing Iran's CO_2 emissions for the period 1990–2010. The Refined Laspeyres Index (RLI) method which was developed by Sun (1998) has been applied to the data set. The data set is gathered from the World Bank database. The impacts of the four main factors, namely economic activity, energy intensity, carbon intensity, and population, have been considered in the analysis.

The structure of the paper is as follows. In the next section, a brief literature review about the country based decomposition analysis studies is provided. In the third section the decomposition analysis methodology and basically the Refined Laspeyres Index method are discussed. This section is followed by the overview of Iran's economic, demographic, energy, and environmental conditions. The empirical results are presented in section 5. The last section contains the conclusions of the paper.

Literature Review

Various methodologies have been developed to analyze the variations in energy and environmental factors. Decomposition Analysis (DA) techniques are among these methodologies used to identify the components changing the energy demand and related CO_2 emissions. A remarkable literature exists about the country-based decomposition studies. For instance, Paul and Bhattacharya's (2004) country-based study, analyzes the factors that increase and decrease the CO_2 emissions from the energy use of India between 1980 and 1996. Their empirical results indicate that economic activity has the greatest increasing effect on CO_2 emissions in India. Fankhauser and Cornillie (2004) studied the decomposition of energy data to identify the determining factors for energy intensity improvement in Eastern Europe and former Soviet Union countries. The researchers proved that energy prices and progress in enterprise restructuring are the two major contributors of efficient energy use. Furthermore, Wang et al. (2005) has also studied on decomposition of the energy-related CO_2 emissions for China between 1957 and 2000. Their empirical findings have shown that China achieved a remarkable decline in its CO_2 emissions due to improvements in its energy intensity. Kawase et al. (2006) have also worked on the decomposition of CO_2 emissions in Japan, for the period between 1985 and 1995, and they evaluated the long-term climate stabilization scenarios. On the other hand, Ma and Stern (2008) have also focused on Chinese CO_2 emissions between 1980 and 2003, and their results revealed that technological improvements reduced energy intensity, and this led to a reduction in overall CO_2 emissions. Moreover, Akbostanci et al. (2009) has studied the decomposition of CO_2 emissions for Turkey between 1970 and 2006 and their study showed that economic activity and energy intensity are the two major factors for evaluation of CO_2 emissions. Difurio (2010) has also focused the decomposition of CO_2 emissions in the US for the period 1990–2004 and their study showed that efficient energy use and reduction of fossil fuels in overall energy consumption offset the accelerating contributions of GDP per capita and population growth in the entire research period. Kumbaroglu's (2011) work has focused on the decomposition of CO_2 emissions for Turkey between the years

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1990 and 2007 by main economic activities, including agriculture, manufacturing, electricity, residential buildings, and transportation. His findings reveal that energy intensity and economic activity are the two major determinants of increasing CO_2 emissions in Turkey. On the other hand, carbon intensity and composition make only minor contributions to CO_2 emissions. Lei and Fan (2015) analyzed the CO_2 emissions in Beijing that are emitted from transportation over 1995 – 2012. By using the Generalized Fisher Index (GFI) method, the authors concluded that the economic growth, energy intensity, and population size have raised carbon emissions in the city during the study period. On the other hand they reported that transportation intensity and energy structure are the two factors that reduced the speed of transportation emissions in Beijing. Rüstemoğlu (2016) has provided a comparative decomposition analysis for Turkey's and Iran's carbon dioxide emissions from 1990 to 2011. By utilizing the Logarithmic Mean Divisia Index (LMDI) technique the researcher concluded that economic activity and population are the two major carbon emissions accelerating factors in Turkey and Iran. In addition, the researcher stated that Turkey has better achievements in terms of energy efficiency. *Table 1* summarizes these selected decomposition analysis studies in the literature.

Table 1. *Selected studies that provide an overview of country based decomposition analysis*

Study	Methodology	Empirical Findings
Paul and Bhattacharya, 2004. CO_2 emissions from energy use in India: A decomposition analysis	• Refined Laspeyres Index Method	• Economic Activity is the major factor which is increasing the CO_2 emissions.
Fankhauser and Cornillie, 2004. The energy intensity of transition countries	• Laspeyres Index Method • Paasche Index • Average Divisia Index Method • Adaptive Divisia Weighting Method	• For efficient energy use, they concluded that energy prices and progress in enterprise restructuring are the two main factors.
Wang et al, 2005. Decomposition of energy – related CO_2 emissions in China: 1957 – 2000.	• Logarithmic Mean Divisia Index Method	• China achieved a success to reduce the speed of CO_2 emissions by energy efficiency.
Kawase et al, 2006. Decomposition analysis of CO_2 emissions in long – term climate stabilization scenarios.	• Extended Kaya Identity • Scenario Analysis	• They evaluated the long term climate stabilization scenarios for Japan.
Ma and Stern, 2008. China's changing energy intensity trend: A decomposition analysis.	• Logarithmic Mean Divisia Index Method	• A decrease is observed in energy intensity due to the technological improvements.
Akbostanci et al, 2009. A decomposition analysis of CO_2 emissions from energy use: Turkish case.	• Logarithmic Mean Divisia Index Method	• Economic activity and energy intensity are the two major contributing factors to Turkish CO_2 emissions.

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Difurio, 2010. A decomposition nalysis of CO_2 emissions in the United States.	• Logarithmic Mean Divisia Index Method	• Efficient energy use and decreasing use of fossil fuels helped to reduce the increasing impact of economic activity and population growth in CO_2 emissions in US.
Kumbaroglu, 2011. A sectoral decomposition analysis of Turkish CO_2 emissions over 1990 – 2007.	• Refined Laspeyres Index Method.	• Major contributing factors: Scale effect and energy intensity. • Minor contributing factors: Carbon intensity and domposition effect.
Lei and Fan, 2015. Decomposition analysis of energy – related carbon emissions from the transportation sector in Beijing.	• Generalized Fisher Index (GFI) method.	• Economic growth, energy intensity and population have raised carbon emissions in the sector. • Energy structure and transportation intensity have followed a decreasing trend in CO_2 emissions in transportation sector.
Rüstemoğlu, 2016. Environmental cost of economic growth: Determinants of CO_2 emissions in Turkey and Iran.	• Logarithmic Mean Divisia Index Method.	• Major determining factors: Economic activity, population. • Better energy efficiency achievements in Turkey as compared to Iran.

Decomposition Analysis Methods

Introduction to Decomposition Analysis

Decomposition analysis techniques can be considered mainly in two groups, Index Decomposition Analysis (IDA) and Structural Decomposition Analysis (SDA). SDA uses the input–output model in quantitative economics; however, IDA uses the index number concept under various methods linked to different index computations (Kumbaroğlu, 2011).

Both SDA and IDA methodologies are utilized in energy and CO_2 decomposition analysis, however, the use of IDA studies regarding CO_2 emissions is much larger. Ang and Zhang (2000) produced a survey of IDA related to the energy and environmental studies. In addition, Hokestra and Van Den Bergh (2003) made a comparison between SDA and IDA techniques and they stated that SDA is more efficient for the decomposition of economic and technological effects and IDA is more efficient for more detailed time and country studies. According to Ma and Stern (2008), the main advantage of IDA is it can be applied to any available data at any level of application. Like any other methods decomposition analysis also have some shortcomings. For instance, the analysis does not include the biomass use, since the comparable international data is not available. Therefore non - CO_2 greenhouse gas emissions are not included. In addition, CO_2 emissions only from fossil fuel combustion are

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considered. The other activities (forestry, land use changes) which also emit CO_2 emissions are not analyzed, again since the comprehensive data for them is not available. However, since it identifies the major contributing factors of CO_2 emissions generally a decomposition analysis is employed in the paper.

Refined Laspeyres Index Method

Different methods have been developed and utilized under the IDA methodology. As Kumbaroglu (2011) states, the Laspeyres Index isolates the impact of a variable by letting that specific variable change between two years while holding other variables as a constant at the base year values. The Laspeyres Index method contains a residual term; therefore, a refinement process is suggested by Sun (1998) to eliminate it. Sun's refinement process distributes the residual term to each variable. This approach is referred to as the Refined Laspeyres Index method and according to Ang and Zhang (2000) it passes all tests, namely time reversal, factor reversal, and zero value robustness. We chose to use the Refined Laspeyres Index method because it is easy for both calculation and understanding and does not leave any residual terms.

The Refined Laspeyres Index method is an extension of the Kaya Identity. The Kaya Identity is used to investigate the role of different factors that change CO_2 emissions (Kumbaroğlu, 2011). It describes the CO_2 emissions as a multiplication of four factors: population (POP), carbon intensity of energy use (CO_2/ENG), energy intensity of production (ENG/GDP), and production per capita (GDP/P). That is:

$$CO_2 = POP * \frac{CO_2}{ENG} * \frac{ENG}{GDP} * \frac{GDP}{POP} \quad \text{(Eq. 1)}$$

For this study, our aim is to show the overall impacts of the main components on CO_2 emissions. Therefore, we calculate the cumulative contributions of the four main components, as follows:

$$\begin{aligned} CO2(i) &= \textstyle\sum_j CO2(j), \\ POP(i) &= \textstyle\sum_j POP(j), \\ ENG(i) &= \textstyle\sum_j ENG(j), \\ GDP(i) &= \textstyle\sum_j GDP(j). \end{aligned}$$

Then the first equation can be written as,

$$CO_2(i) = \textstyle\sum_j POP(j) * \frac{CO2(j)}{ENG(j)} * \frac{ENG(j)}{GDP(j)} * \frac{GDP(j)}{POP(j)} \quad \text{(Eq. 2)}$$

It is possible to denote the carbon intensity by CI, the energy intensity by EI, and economic activity by EA. Then the previous equation can be written as:

$$CO_2(i) = \textstyle\sum_j POP(j) * CI(j) * EI(j) * EA(j) \quad \text{(Eq. 3)}$$

The effect of changes in production activity is referred to as the economic activity effect (EA_i^t), and it can be calculated as:

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Economic Activity Effect

$$(EA_i^t) = \sum_j \Delta EA(j) * \left\{POP(j) * EI(j) * CI(j) + \frac{1}{2} * (\Delta POP(j) * EI(j) * CI(j) + POP(j) * \Delta EI(j) * CI(j) + POP(j) * EI(j) * \Delta CI(j))\right\} + \sum_j \Delta EA(j) * \{\frac{1}{3} * \left(\Delta POP(j) * \Delta EI(j) * CI(j) + \Delta POP(j) * EI(j) * \Delta CI(j) + POP(j) * \Delta EI(j) * \Delta CI(j)\right) + \frac{1}{4} * (\Delta POP(j) * \Delta EI(j) * \Delta CI(j))\} \quad \text{(Eq. 4)}$$

The economic activity effect (EA_i^t) shows the change in CO_2 emissions results from changing activity levels. According to the economic activity effect, an increase in activity levels increases the amount of CO_2 emissions, and a decrease in activity levels decreases the amount of CO_2 emissions.

Population Effect

$$(POP_i^t) = \sum_j \Delta POP(j) * \{EA(j) * EI(j) * CI(j) + \frac{1}{2} * (\Delta EA(j) * EI(j) * CI(j) + EA(j) * \Delta EI(j) * CI(j) + EA(j) * EI(j) * \Delta CI(j))\} + \sum_j \Delta POP(j) * \{\frac{1}{3} * \left(\Delta EA(j) * \Delta EI(j) * CI(j) + \Delta EA(j) * EI(j) * \Delta CI(j) + EA(j) * \Delta EI(j) * \Delta CI(j)\right) + \frac{1}{4} * (\Delta EA(j) * \Delta EI(j) * \Delta CI(j))\} \quad \text{(Eq. 5)}$$

The population effect (POP_i^t) shows the change in CO_2 emissions results from changes in the population. An increase in population increases CO_2 emissions and a decrease in population decreases CO_2 emissions.

Energy Intensity Effect

$$(EI_i^t) = \sum_j \Delta EI(j) * \left\{EA(j) * POP(j) * CI(j) + \frac{1}{2} * \left(\Delta EA(j) * POP(j) * CI(j) + EA(j) * \Delta POP(j) * CI(j) + EA(j) * POP(j) * \Delta CI(j)\right)\right\} + \sum_j \Delta EI(j) * \{\frac{1}{3} * (\Delta EA(j) * \Delta POP(j) * CI(j) + \Delta EA(j) * POP(j) * \Delta CI(j) + EA(j) * \Delta POP(j) * \Delta CI(j)) + 1/4 * (\Delta EA(j) * \Delta POP(j) * \Delta CI(j))\} \quad \text{(Eq. 6)}$$

The energy intensity effect (EI_i^t)suggests an indication of efficiency in the energy process, conversion technologies, and energy conservation. Energy-saving activities, reducing the use of fossil fuels and the use of renewable technologies increase energy efficiency. As a result, energy efficiency reduces the amount of CO_2 emissions.

Carbon Intensity Effect

$$(CI_i^t) = \sum_j \Delta CI(j) * \left\{EA(j) * POP(j) * EI(j) + \frac{1}{2} * \left(\Delta EA(j) * POP(j) * EI(j) + EA(j) * \Delta POP(j) * EI(j) + EA(j) * POP(j) * \Delta EI(j)\right)\right\} + \sum_j \Delta CI(j) * \{\frac{1}{3} * (\Delta EA(j) * \Delta POP(j) * EI(j) + \Delta EA(j) * POP(j) * \Delta EI(j) + EA(j) * \Delta POP(j) * \Delta EI(j)) + 1/4 * (\Delta EA(j) * \Delta POP(j) * \Delta EI(j))\} \quad \text{(Eq. 7)}$$

The carbon intensity effect (CI_i^t) is used to show the impact of fuel substitution on CO_2 emissions. For instance, if the share of renewable sources increases or if people use natural gas instead of coal, there will be a decline in overall CO_2 emissions.

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The change of CO_2 emissions between two years is the sum of these four effects:

$$\Delta CO_2(i) = Economic\ Activity(i) + Population(i) + Energy\ Intensity\ (i) + Carbon\ Intensity(i) \quad \text{(Eq. 8)}$$

For a detailed analysis regarding the RLI method, Ang and Zhang's (2001) work could be followed.

Analysis of Data

The decomposition analysis covers the period 1990–2010, and the energy, CO_2, economic activity, and population data sets employed in this study are taken from the World Bank database. An overview of the economic and demographic developments, energy market, and emission growth trajectories is presented below. [1]

Economic and Demographic Developments in Iran

During 1990–2010, real GDP grew at an annual average rate of 4.5 %. However, Iran's economy was faced by recession in the years 1993 and 1994 and the real GDP declined by 1.6 % and 0.4 % (World Bank, 2015). Recently, economic sanctions, which are targeting the oil and natural gas sectors, hampered Iran's economy profoundly (EIA, 2015). Starting from 2008 the economic growth of Iran slowed down and in 2012, real GDP declined by 1.9 %. Real GDP per capita also increased from $1801 to $3259 during the research period (World Bank, 2015).

Iran's population has increased from 56.3 million (in 1990) to 74.4 million (in 2010). The average population growth rate of Iran is 1.4 % and this value is very similar to the world's population growth rate (World Bank, 2015). The share of the urban population also increased from 56.3 % (in 1990) to 68.9 % (in 2010) (World Bank, 2015).

Energy Market in Iran

Iran is the 12th largest energy consumer in the world and during the study period its energy consumption has increased from 69.3 thousand kt to 210.6 thousand kt (World Bank, 2015). Similarly, the energy production of Iran has increased rapidly from 187.8 thousand kt to 350.1 thousand kt between 1990 and 2010. According to EIA (2015), Iran is the fourth largest oil producer and second largest natural gas producer in the world and 98 % of overall energy demand is generated from these sources. The electricity production and electricity consumption of the country is 3.9 times and 3.7 times greater in 2010 if we compare with the 1990 level. As the World Bank data indicates, 95.8 % of overall electricity demand was generated by nonrenewable energy sources in 2010 (World Bank, 2015). In 2011, Iran's first nuclear energy power plant became operational.

CO_2 Emissions in Iran

Iran's overall CO_2 emissions increased from 211 thousand kt (in 1990) to 571 thousand kt (in 2010) and the country follows the most developed countries such as the US, Germany, Japan, and the UK and the rapidly developing populous countries such as

[1] The data set is available for the interested researchers through the authors' e-mail.

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China and India in the emission rankings (World Bank, 2015). Similarly, per capita emissions of the country have increased from 3.7 tons (in 1990) to 7.7 tons (in 2010). In 2010, shares of the liquid and gaseous fuels in CO_2 emissions are equivalent to 43.2 % and 51.4 % respectively (World Bank, 2015).

Results and Discussion

Before proceeding to the results of the RLI method, we discussed the general structure of Iran's economy, energy market, and CO_2 emissions between 1990 and 2010. The impacts of all identified factors on CO_2 emissions during 1990–2010 can be followed in *Figure 1*.

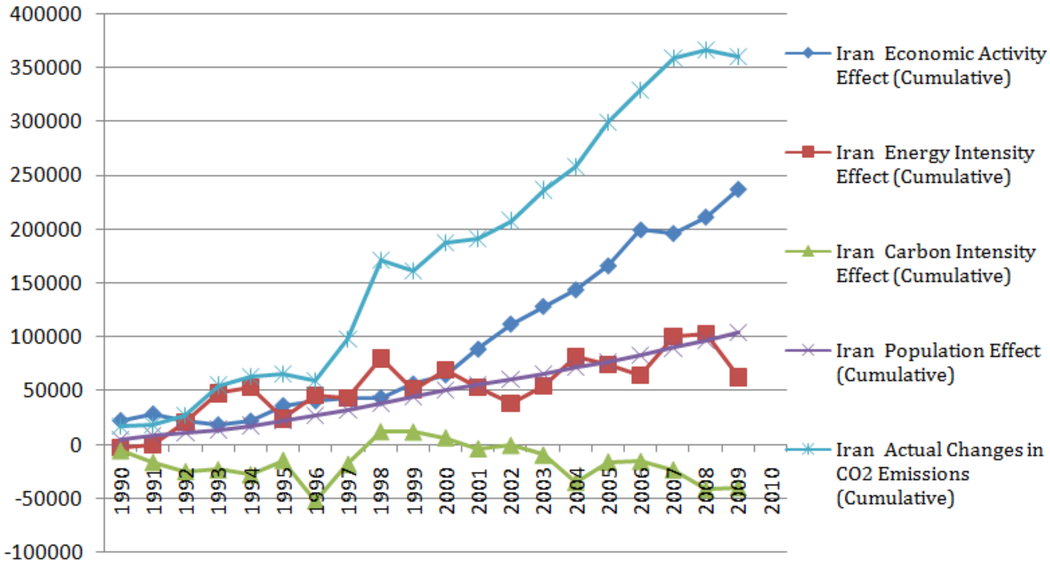

***Figure 1.** Decomposition of CO_2 emissions in Iran between 1990 and 2010*

Figure 1 indicates that the major determinant of Iran's CO_2 emissions is the economic activity. The economic activity is followed by the energy intensity and population. Finally, the carbon intensity has some minor reducing effects on CO_2 emissions in Iran. The detailed analysis about the impacts of the identified factors on CO_2 emissions is presented below.

Economic Activity Effect

As Kumbaroglu (2011) states, the economic activity effect represents the changes in CO_2 emissions resulting from changing economic activities. Except for the years 1993 and 1994, real GDP in Iran has increased. During the period 1990–2010, Iran's GDP and GDP per capita have experienced 139.1 % and 80.9 % increases respectively (World Bank, 2015). As a result of the spectacular economic growth, the economic activity played a dominant role in the decomposition of CO_2 emissions. In the first decade, we observed that the share of economic activity is 34.5 % in overall CO_2 emissions. Moreover, in the second decade, the contribution of economic activity in CO_2 emissions became more visible. The share of the economic activity in CO_2

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emissions has increased to 65.6 % between the years 2000 and 2010. *Figure 2* represents the contribution of economic activity regarding CO_2 emissions.

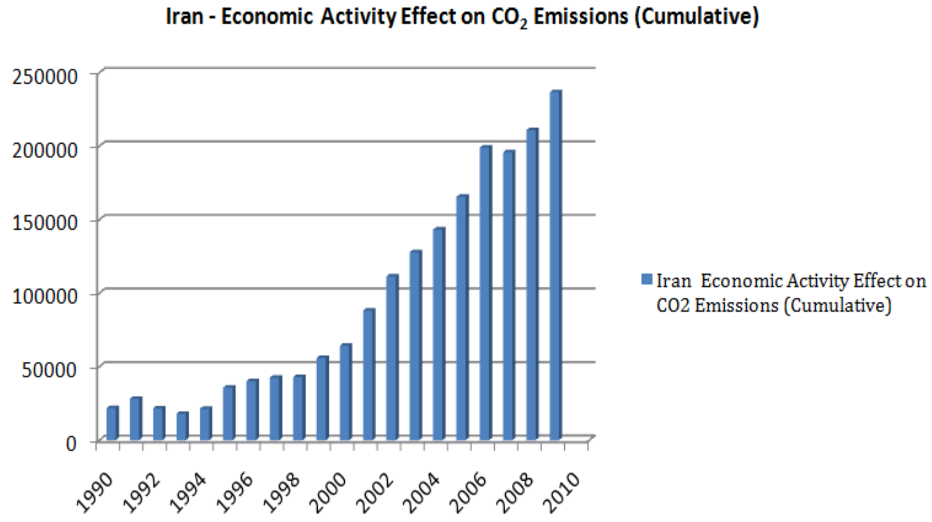

Figure 2. *Contribution of the economic activity to CO_2 emissions in Iran over 199–2010*

Energy Intensity Effect

According to Kumbaroglu (2011), the energy intensity effect provides an indication of the efficiency in energy processes, conversion technologies, and energy conservation. Increasing energy efficiency decreases the CO_2 emissions and vice versa. An energy efficiency goal can be obtained either by using renewable energy sources or reducing the use of fossil fuels by implementing the appropriate energy-saving policies. The energy intensity is the second determining factor on CO_2 emissions in the first decade. The share of the energy intensity is calculated as 31.3 % in overall CO_2 emissions. However, the energy intensity followed a negative trend in the years 1991, 1996, 1998, and 2000. In the second decade the contribution of the energy intensity in CO_2 emissions started to decrease. The share of energy intensity is calculated as 17.1 % in CO_2 emissions between 2000 and 2010. In addition, the energy intensity followed a negative trend in the years 2002, 2003, 2006, 2007, and 2010. The decomposition analysis results indicate that Iran achieved a success in reducing the energy intensity especially in the second decade, however, this success cannot be considered as remarkable since the cumulative contribution of the effect is still positive on CO_2 emissions. The energy intensity effect offsets the accelerating impact of other components when its cumulative impact on CO_2emissions turns to negative. *Figure 3* shows the contribution of the energy intensity effect in CO_2 emissions.

Population Effect

The population effect describes the changes in CO_2 emissions due to the increases or decreases that are observed in the population. Iran's population accelerated to 74.4 million from 56.3 million in 20 years, which corresponds to a 32.1 % increase (World Bank, 2015). As a result of its remarkable growth, population increased CO_2 emissions constantly in the entire research period. Between 1990 and 2000, population

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is the third major determining factor of CO_2 emissions and its share is calculated as 26.9 %. However, during the second decade, the population effect became the second major determining factor on CO_2 emissions and its share has increased to 28.3 %. Since the population of Iran is increasing, then accelerating CO_2 emissions due to the population effect is an expected and a consistent result. *Figure 4* indicates the impact of population effect in Iran's carbon emissions from 1990 to 2010.

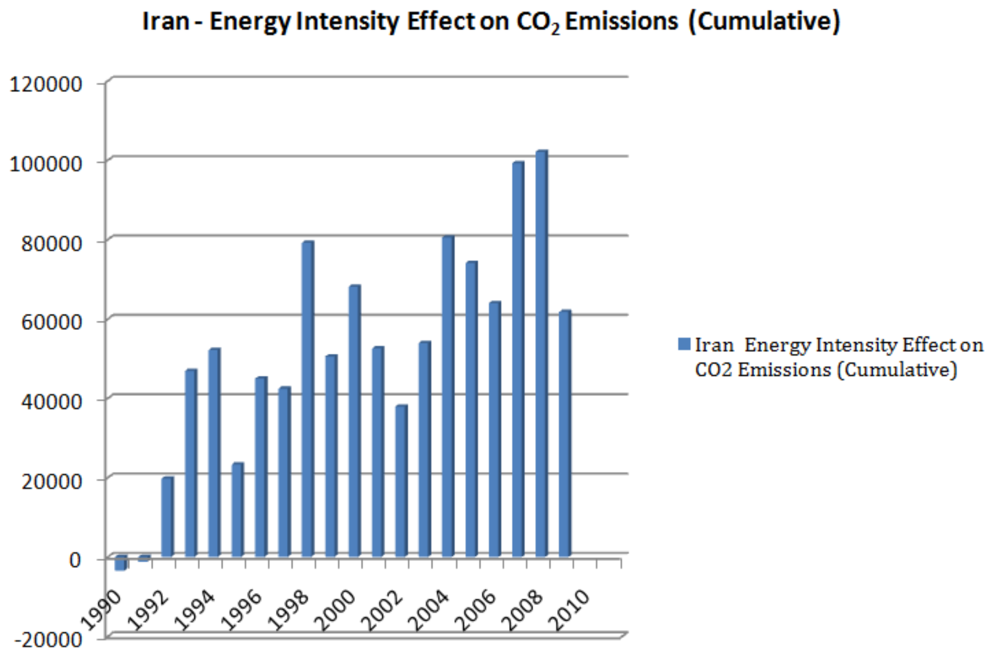

Figure 3. *Contribution of the energy intensity on CO_2 emissions in Iran over 1990–2010*

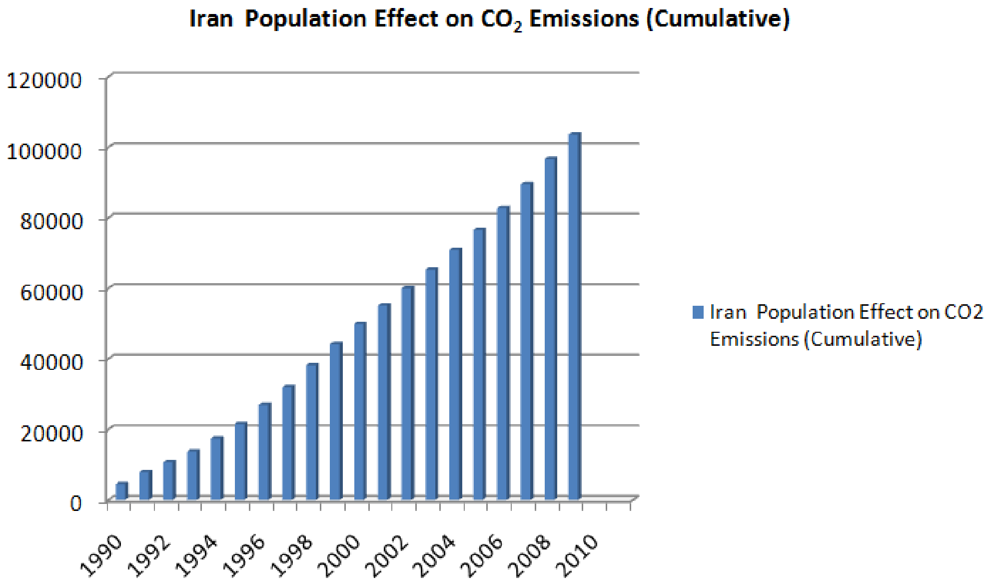

Figure 4. *Contribution of population on CO_2 emissions in Iran over 1990–2010*

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Carbon Intensity Effect

The carbon intensity effect has been used to estimate the impact of fuel substitution in CO_2 emissions. If the energy mix becomes less carbon intensive or renewable sources are implemented instead of nonrenewable sources, then emission reduction can be obtained (Kumbaroğlu, 2011). The share of the carbon intensity in overall CO_2 emissions was 7.2 % during the first decade. Additionally, in the years 1991, 1992, 1993, 1995, 1997, and 2000 the carbon intensity followed a decreasing trend. Furthermore, between 2000 and 2010, the overall contribution of the carbon intensity on CO_2 emissions turned to negative. The share of the carbon intensity is estimated at -11 %, which is a desirable result for a reduction of the emissions. In the second decade, the carbon intensity decreased during the years 2001, 2002, 2004, 2005, 2008, and 2009. This remarkable decline is probably the result of a decreasing share of oil and increasing share of natural gas in overall energy consumption. For instance, in electricity production, the share of oil declined from 37.1 % to 19.8 % and the share of natural gas increased from 52.5 % to 75.9 % for the entire period (World Bank, 2015). The pollution coefficient of natural gas is relatively smaller than the pollution coefficient of oil. The carbon intensity is the only factor that offsets a small amount of the negative contributions of the income effect, the energy intensity effect, and the population effect for Iran's CO_2 emissions. *Figure 5* indicates the declining trend of carbon intensity in CO_2 emissions.

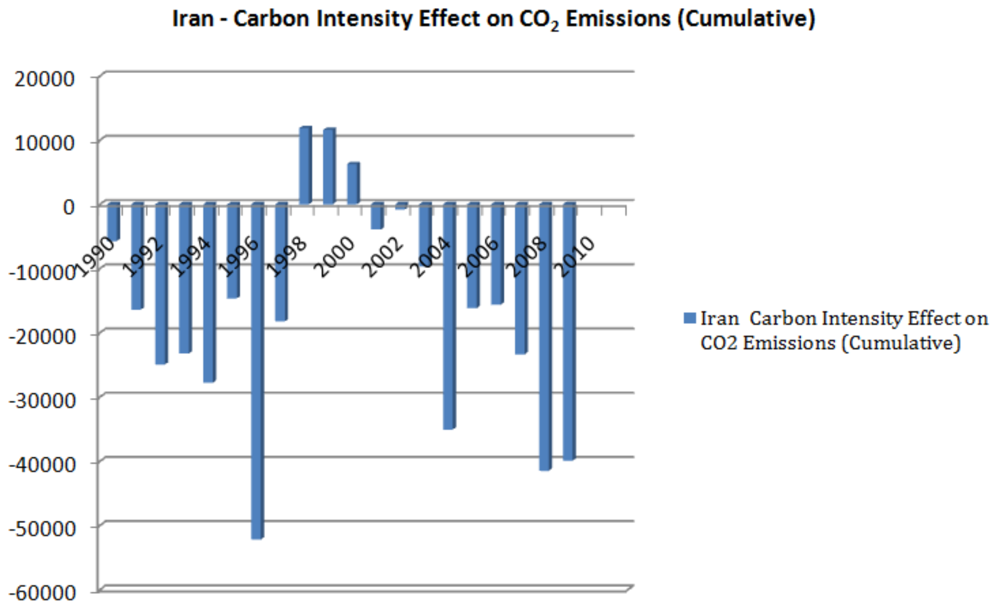

***Figure 5.** Contribution of carbon intensity on CO_2 emissions in Iran over 1990–2010*

Conclusion

In this paper, a decomposition analysis is used to identify the factors that influence the changes in Iran's CO_2 emissions for the period 1990 – 2010. The factors that lead to changes in CO_2 emissions are economic activity, energy intensity, population, and carbon intensity. A particular decomposition technique – the Refined Laspeyres Index method – is used to evaluate the relative contribution of these components that account for changes in CO_2 emissions.

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Our empirical results have shown that in Iran economic activity was the most important component of CO_2 emissions change during 1990–2010. Energy intensity also increases CO_2 emissions; however, its contribution in the second decade was relatively smaller than in the first decade. Population is also a dominant factor on Iran's CO_2 emissions changes; it constantly increased the amount of CO_2 emissions during the study period. Especially in the second decade the contribution of population on CO_2 emissions became more visible. The carbon intensity made minor increasing contributions to CO_2 emissions between 1990 and 2000. However, in the second decade its impact on CO_2 emissions turned to negative, which is a desirable result for emissions reduction. The decrease in the carbon intensity is probably an outcome of the fuel switching. In Iran, during the period 1990–2010 the share of natural gas consumption gradually increased while the share of oil gradually decreased. Since the pollution coefficient of natural gas is relatively smaller than the coefficient of oil, then this led to a reduction in emissions due to the carbon intensity.

Regarding decomposition analysis, Iran is not a widely studied country. Therefore, in this respect, our study contributes to the literature by analyzing Iran. In the future, GDP and population of the country are expected to increase and, therefore, economic activity and population will raise the CO_2 emissions. However, the emission reduction goal can be accomplished by reducing the contributions of energy intensity and carbon intensity. This study creates an opportunity for policy makers to develop economically and environmentally sustainable projects.

Given that Iran is indeed an upper-middle income country which is dependent on oil revenues, volatility in oil prices would create a distortionary and direct impact on the economy. Our policy suggestions include a massive diversifications of the economy to other equally productive sectors; particularly agriculture and industry. Also a very robust and consistent policy framework should be directed towards energy conservation principles in every economic sector. This can be achieved through a high-tech mechanization including the use of fuel efficient equipments in the agricultural sector, use of heat insulators and double layer glasses, and sealing doors and windows in homes and the provision of small loans to households will encourage them to purchase a more energy efficient appliances. In the transportation sector policies should equally be directed not only towards a reduction in gasoline subsidies to bring down CO_2 emissions; but also setting appropriate policies that adheres to energy efficient manufacturing standard vehicles.

Finally since Iran has a remarkable high solar, wind and hydro energy potential due to its geographic location, a policy that is directed towards energy principles will lead to a richer Iran in the near future.

This study made a decomposition analysis to identify the factors that contributed the most to CO_2 emissions in Iran. A further research would be to employ an econometric analysis as of Halicioglu (2009) to include the effect of foreign trade in the determining factors.

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SUBLETHAL TOXICITY OF A DELTAMETHRIN-BASED INSECTICIDE IN RAINBOW TROUT, *ONCORHYNCHUS MYKISS* (WALBAUM, 1792) EMPHASIZING ON THYROID HORMONE RECEPTORS

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Abstract. Metrodesis, a deltamethrin containing commercial formulation has been extensively used in Turkey, in the framework of integral pest management thanks to its target-specific toxicity. So as to assess the potential impacts of Metrodesis (EC; active ingredient 25 g/L of deltamethrin) on thyroid hormone (TH) homeostasis of rainbow trout (*Oncorhynchus mykiss*); mRNA levels of thyroid hormone receptors (*TR*s), *TRα* and *TRβ*, were determined by RT-PCR soon after sublethal administration in a static bio-assay system. The sublethal dose of 4.15 µg/L (¼ of LC_{50} value) led to induction of relative expression levels of *TRα* and *TRβ* genes both in muscle and liver tissues, provided that *TRα* expression quantities were considerably higher than *TRβ*, although the differences were found statistically insignificant (P>0.05). Results of this study revealed an endocrine interaction between deltamethrin-based formulation and TH homeostasis corroborating previous researches that approved deltamethrin as an endocrine disrupting compound.
Keywords: *fish, bioassay, synthetic pyrethroid, gene expression, mRNA*

Introduction

A great deal of chemical substances dumped to the aquatic environment disrupt endocrine homeostasis in organisms by interfering with developmental processes and endocrine systems (Mortensen et al., 2006). Endocrine disrupting chemicals (EDCs) are endocrine active compounds causing specific effects on endocrine systems at several levels without relevant toxic actions (Kloas, 2002; Kloas and Lutz, 2006). In respect to the European Union (EU) list of potential EDCs, pyrethroids are candidate substances as compiled in category 1 (Danish EPA, 2016).Pyrethroids are synthetic analogs of pyrethrins, which are toxic components contained in the flowers of *Chrysanthemum cinerariaefolium* (De Assis et al., 2009). Deltamethrin [(S)-α-cyano-3-phenoxybenzyl, (1R)-cis-3-(2,2-)-2,2 dimethycyclopropane carboxylate], the active ingredient of the commercial formulation Metrodesis, is a non-composite synthetic pyrethroid, which is used in control of ectoparasites for animals, as an insecticide in agricultural production and to manufacture long-lasting insecticidal mosquito nets for controlling malaria vectors (Siwicki et al., 2010; Sayeed et al., 2003).

Considering its high efficiency of counteracting the insects, as well as non-cumulative characteristic, selective toxicity and rapid photodegradation (Benli et al., 2009), as in other pyrethroids, it has wide acceptability (Erdoğan et al., 2011; Haverinen and Vornanen, 2014; Haverinen and Vornanen, 2016). It is commercially available as

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emulsifiable concentrate for water emulsion and extensively used in Turkey for pest management. In fish, an ectothermic vertebrate, deltamethrin emanated extremely high toxicity (Haya, 1989; Bradbury and Coats, 1989; Viran et al., 2003; Mikata et al., 2011; Srivastav et al., 2010). This is comparatively associated with lower metabolic rate of fish and ineffective metabolic degradation of this neurotoxic agent by fish tissues (Haverinen and Vornanen, 2014; Haverinen and Vornanen, 2016) due to the lack of carboxylesterases (Haya, 1989) that catalyze hydrolysis of pyrethroids in the liver (Srivastav et al., 1997). Furthermore, the lipophilic structure of deltamethrin entails the high rate of gill absorption which is an another remarkable factor to elucidating the high sensivity of fish to toxicity (Viran et al., 2003; Mikata et al., 2011; Srivastav et al., 2010).Some critical toxic effects of deltamethrin on fish can be varied with respect to species, age of the fish and duration of the exposure and elaborated as cardiotoxicity (Haverinen and Vornanen, 2014), disturbance of the calcium and phosphate homeostasis (Srivastav et al., 1997), changes in liver, gill and gut histopathology (Cengiz and Unlu, 2006), inducing hyperglycemia (Aziz et al., 2009) and significant alterations in blood chemistry and kidney function (Aziz et al., 2009; Kumar et al., 1999). It is also noteworthy that, deltamethrin acts as a oxidative stressor and causes inhibition of activity in various proteins including antioxidant enzymes (Erdoğan et al., 2011) and inducing the expression of *HSP70* (Ceyhun et al., 2010b), *CYP 1A*, *MT-A, MT-B* (Erdoğan et al., 2011) and reducing *IGF*s and *GH* in rainbow trout (Aksakal et al., 2010). Although some studies has been done on the impacts of deltamethrin-based formulations (Decis) on avians and fish (Marques et al., 2014; Bhaskar et al., 2016), nevertheless, there are limited researches related to the endocrine disrupting potential of this chemical on fish (Brander et al., 2016; Tu et al., 2016) when we consider the extensive use of this commercial formulations for the pest management.

The thyroid hormones (THs; T_4, T_3) regulate a wide range of cellular functions, including growth, development, differentation, metabolism and maintenance of homeostasis, in almost every tissue of teleost (Brent, 1996; Marchand et al., 2001; Filby and Tyler, 2007). Regulation of gene expression by TH is mediated by nuclear thyroid hormone receptors (*TR*s) (Wu and Koenig, 2000) which has two forms (*TRα* and *TRβ*) encoded by distinct genes (Filby and Tyler, 2007). As presented in Kloas and Lutz (2006), thyroid system of fish is affected direct or indirectly via accumulation via exposure or the food chain by endocrine disrupters and a detailed understanding of hypothalamus-pituarity-thyroid (HPT) axis regulation in teleosts is necessary to whole evaluation of thyroid-disrupting impacts of environmental compounds.

Therefore, the present paper aims to reveal the endocrine disruption potential of commercially available insecticide of deltamethrin (Metrodesis) in a well-known teleost, *Oncorhynchus mykiss*, centering upon the TH mechanism regarding to *TR*s as a response to sublethal exposure.

Materials and Methods

Insecticide

The commercial formulation tested, Metrodesis, is manufactured by Özbaşkent Kimya, Ankara, Turkey. It is an emulsifiable concentrate formulation containing 25 g deltamethrin/L in light aromatic solvent naptha (petroleum>25%).

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Experimental design

Rainbow trout (*Oncorhynchus mykiss*) (mixed sex) with a mean body weight of 83.36±7.73 g (Mean±S.D., *n*=252) were obtained from Cifteler Sakaryabasi Aquaculture and Research Station of Ankara University in Eskişehir, Turkey where the experiments were conducted. Prior to experiments for acclimatization, fish were kept in 200 L tank, aerated consistently, for two weeks and fed daily with commercial trout feed (45% crude protein) ad libitum. Fish were deprived of feed for 48 h before and during the experiment. To eliminate the fish faeces and excess feed, the water in the tanks were changed (10%) by siphoning and replaced with fresh water in the acclimatization period. All tanks were shielded with netting material to prevent fish from escape and minimize stress during the trials. The maintenance of the animals and the experiments were performed under approved protocols in accordance with the principles of Ankara University Animal Ethics Committee (Date/No: 16.02.2011/2011-105-384). In exposure system, providing triplicates per each concentration (fish per tank; preliminary, main and control: 4 / sublethal and control: 10), dosing solutions of deltamethrin were prepared from Metrodesis and the calculation of dosing solutions was done according to the active ingredient of insecticide. The dosing volumes never exceeded 0.2 ml.

Water analysis

The water quality was evaluated (APHA, 1995) and the measurements of physicochemical parameters of water during the preliminary (range finding), main (LC_{50}) and sublethal toxicity tests were presented in *Table 1*.

Table 1. *The physicochemical parameters of water during the preliminary (P), main (M) and sublethal (S) toxicity tests (Mean values±S.D.) (n=3).*

Parameters	**P**	**M**	**S**
Dissolved oxygen (mg/L)	6.36±0.01	6.45±0.06	6.48±0.03
pH	6.93±0.05	7.21±0.10	7.26±0.08
Temperature (°C)	19.60±0.80	20.10±0.60	20.00±0.40
Electrical conductivity (µS/cm)	309.30±15.60	416.30±9.70	415.70±5.20
Alkalinity (mg/L $CaCO_3$)	40.00±0.00	40.00±0.00	40.00±0.00
Hardness (mg/L $CaCO_3$)	56.80±2.00	46.00±5.30	28.00±8.50
NH_3-N (mg/L)	0.65±0.12	0.98±0.02	0.83±0.04
NO_2-N (mg/L)	0.01±0.00	0.01±0.00	0.01±0.00
NO_3-N (mg/L)	1.42±0.26	1.36±0.20	1.29±0.30

Acute toxicity (LC_{50} –Median lethal concentration)

Acute toxicity tests were carried out in compliance with the standardized methods (OECD, 1992) at two stages in unrenewable static experimental conditions for 96 h with a total number of 192 fish. In stage I, a range finding (preliminary) test was performed

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to determine the concentrations of the main acute test by using wide ranges (Schäperclaus, 1979) as follows, 0.5, 1, 5, 10, 50, 100, 200, 300 µg/L of dosing concentrations. In stage II, with regard to the preliminary test results, six concentrations (4, 8, 10, 16, 32 and 50 µg/L) that comprised 0% and 100% death were chosen as the main acute toxicity concentrations. Meanwhile, control groups (non-exposure) were maintained for both preliminary and main exposure tests in triplicates.In the course of exposure tests, dead individuals were removed immediately from the tanks and behavioral changes and mortalities were monitored closely and noted down every 24 h. The Probit regression, a dose-response, analysis was used to compute the median lethal concentration (LC_{50}) of deltamethrin, with confidence limit of 95%, for 96 h exposure (Unsal, 1996).

Sublethal toxicity

For the assessment of sublethal effects of Metrodesis (25 g deltamethrin/L), ¼ of 96 h LC_{50} value as computed in acute toxicity test, was considered and exposed as a sublethal concentration. The sublethal exposure tests were conducted in triplicates for both treatment and control groups with total number of 60 fish in static system likewise in acute toxicity tests.

Sampling of fish tissues

At the end of the sublethal exposure of deltamethrin, the tissue samples (liver and white muscle) of rainbow trout were collected from all individiuals of both exposure and control groups. The dissection of liver and muscle tissues was performed on crushed ice and the samples were flash-frozen in liquid nitrogen and preserved at -80°C for further procedures.

RNA isolation and reverse transcription into cDNA

Total RNA was isolated from the frozen tissues using High Pure RNA Tissue Kit (Roche Diagnostics) according to the manufacturer's instructions. The purity of isolated RNA was quantified by spectrophotometric absorbance (A_{260}/A_{280} ratio) using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany) and the integrity of RNA samples was verified by agarose gel electrophoresis on agarose gel that comprised ethidium bromide (EB).The complementary DNA (cDNA) was synthesized by reverse transcribing from total RNA using the transcriptor first strand cDNA Synthesis Kit (Roche Diagnostics) following the protocol of the manufacturer. Random hexamer primer was chosen in reverse transcribing of cDNA due to the use of *18S* rRNA as a housekeeping gene. The cDNAs were stored at -20°C until used for real-time PCR (RT-PCR).Gene-specific primer pairs and probes were provided from Universal ProbeLibrary (UPL) system (Roche Diagnostics) which constituted a web-based software (ProbeFinder software). UPL probes are hydrolysis probes substituted with Locked Nucleic Acids (LNA) and they are labeled at the 5′ end with FAM (Fluorescein amidite) and at the 3′ end with a dark quencher dye. The sequences of primer pairs and probes were presented in *Table 2*.

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Table 2.*Primer and probe sequences of reference (housekeeping) and target genes of 18S, TRα, TRβ respectively, in rainbow trout (Oncorhynchus mykiss).*

Gene	Primer/Probe	Sequence	Gene Bank Accession No.
18S	Forward primer	ATGGTCTAACATCTTATAAGCGGCTT	AF308735.1
	Reverse primer	GCGCAGAGAAGTTGACTGG	
	UPL*(#118, cat.no. 04693523001)	CACTGGGA	
TRα	Forward primer	GAGAAGAGGAAGAAGGAGGAGAT	AF302245.1
	Reverse primer	GAACTTGCGTTTCTGTTTCCA	
	UPL (#24, cat.no. 04686985001)	CAGCTCCC	
TRβ	Forward primer	GAGGCCCACATGTCAACTAAC	AF302246.1
	Reverse primer	TGGTTTCCTTCACCCCCACT	
	UPL (#37, cat.no. 04687957001)	TGCCCTGG	

*UPL; Universal ProbeLibrary Probe

RT-PCR procedure

The amplification was performed with a LightCycler 480 RT-PCR system (Roche, Switzerland) using LightCycler 480 Probes Master Kit (Roche Diagnostics). The thermal cycling conditions consisted of an initial pre-incubation for 10 min at 95°C, followed by 45 cycles of denaturation of the target DNA at 95°C for amplification of the target DNA with for 10 s, primer annealing at 60°C for 30 s, extension at 72°C for 1 s and 1 cycle of cooling for 30 s at 40°C.A standart curve was constituted for target and housekeeping gene seperately and efficiency of each reaction (1.8-2.0) was determined. PCR samples were run in duplicates for standarts, samples and negative controls. Negative control samples (nuclease-free water instead of cDNA) were used in each run to test the target specifity of the cDNA amplification.

Data normalization

The housekeeping gene *18S* was used as an endogenous standart and expressions of target genes of *TRα* and *TRβ* were normalized to the corresponding level of *18S* mRNA relatively to the untreated control by using comparative Ct ($2^{-\Delta\Delta C_T}$) method (Livak and Schmittgen, 2001).

Statistics

All data were expressed here as means±standart error of the mean (SEM) unless indicated otherwise. A total of ten fish were used from exposure and control groups and two technical replicates per fish and treatment were made. Paired Samples T-test, a confidence level of 95%, was applied using statistical software IBM SPSS Statistics 23.0 to determine the presence of significant differences among the groups.

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Results

Median lethal concentration (LC_{50})

The calculated 96-h acute LC_{50} value of Metrodesis (25 g deltamethrin/L) in a static bioassay system for rainbow trout was 16.582 µg/L (χ^2=0.500) with 95% confidence limits of 13.525-22.156 using Probit regression analysis. The sublethal concentration was 4.15 µg/L and determined as ¼ of 96-h LC_{50} value. The behavioral responses of the individuals during the acute exposure were observed and recorded every 24-h. In the highest concentration (50 µg/L), within an hour, test fish were demonstrated reactions including movements as trembling and fasciculation, poising at upright, tendency to swim to the water surface and gulping for air. At all concentrations, excessive mucus secretion was detected and below 50 µg/L, fish were quite motionless and mostly gathered at the edge of the tanks with high respiration frequency. Control test groups were comparatively calm and no significant behavioral changes were monitored.

Expression of TRs in white muscle and liver

The relative expression levels of *TRα* and *TRβ* genes in white muscle and liver tissue of rainbow trout after exposed to sublethal treatment of deltamethrin (4.15 µg/L) were presented in *Table 3*. Sublethal exposure of deltamethrin did not emerge a statistical difference (P>0.05) in expression of *TRα* gene between white muscle and liver tissue, however a slight but insignificant up-regulation was observed in liver tissue compare to white muscle. Similarly, no significant mRNA alteration (P>0.05) was detected for *TRβ* of white muscle and liver tissue with respect to the toxic treatment. Unlikely from *TRα* expression pattern, the white muscle tissue was slightly up-regulated for *TRβ* gene, though the difference was nominal. However, when we compare the results in a gene-specific manner, for *TRα* gene, an apparent increase of mRNA in the white muscle, which was about 5 fold, was recorded compare to liver tissue, whereas the liver tissue was 6 fold higher than white muscle. In general, the relative expression quantity of *TRα* gene was found considerably high in comparison to *TRβ* for both white muscle and liver tissue due to the 96-h sublethal exposure of deltamethrin. Nonetheless, no statistical differences were noted (P>0.05) according to Paired samples T-Test, although a remarkable effect was observed corresponding to deltamethrin treatment

Table 3.*mRNA expression levels of TRα and TRβ genes in white muscle and liver tissue of rainbow trout that are expressed relative to control values and normalized to 18S with their respective standard errors in response to 4.15µg/L of deltamethrin.*

Gene	**Fish Tissue**	
	White muscle	**Liver**
TRα	3.036±1.57$^{N.S.*}$	3.129±1.63
TRβ	0.679±0.16	0.578±0.19

*N.S. (Non-significant); Means (Means±S.E.M) (n=5) in the same row and column were found in-significant according to Paired Samples T-test (P>0.05).

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Discussion

Deltamethrin, a synthetic pyrethyroid, is well-known with its rapid knock down action in insects, low persistence in the environment and low mammalian toxicity (Erdoğan et al., 2011). Despite these aspects and wide spread on a global scale, it has become the most controversial environmental compound due to the potential of high toxicity to aquatic organisms notably to fish. Here, the 96 h acute LC_{50} value of commercial formulation of deltamethrin, Metrodesis, in rainbow trout was found 16.582 µg/L. In quite a few research have been reported the LC_{50} of deltamethrin in a variety of fish and their findings, which are consistent with the present study result, revealed the potential of high toxicity of this substance to fish. Ural and Sağlam (2005) stated that incremental concentrations of deltamethrin exposure, duration of 96 h, led to increase the number of death fry fish in rainbow trout, that the estimated LC_{50} value was 0.6961 µg/L. Similar observations were notified for common carp by Koprucu and Aydın (2004) after 48 h exposure and the LC_{50}values were 0.213 and 0.074 µg/L for embryos and larvae, respectively. The toxicity takes effect in a time, dose, specie and the size manner as so in young mirror carp that the LC_{50} values were found 9.41, 4.47, 2.37 and 1.65 µg/L in the time of 24, 48, 72 and 96 h of exposure in a static bioassay system (Calta and Ural, 2004). In the same work, the toxicity studies for deltamethrin compiled in a table for *Poecilia reticulata* (5.13 µg/L for 48 h), *Clarias gariepinu*s (40.01 µg/L for 96 h), *Esox lucius* (23.0 µg/L for 96 h) and *Ctenopharyngodon idella* (91.0 µg/L for 96 h). The other LC_{50} studies in *Oreochromis niloticus* as reported by Golow and Godzi (1994) and Boateng et al. (2006) resulted with values of 14.5 and 15.47 µg/L, these results were comparable with the present data even in the case of different species. The exposure with deltamethrin revealed behavioral changes in the treatment groups, in congruent of the present study, Ural and Saglam (2005) also monitored some responses in *O. mykiss* as loss of equilibrium, hanging vertically, erratic swimming, air gulping from the water surface, staying motionless and color darkening in the concentrations of 1, 2, 4, 6, 8, 10, 12, 25 and 50 µg/L, and no changes in the control group. Similar changes were detected in *P.reticulata*(Viran et al., 2003),*Cyprinus carpio*(Koprucu and Aydın, 2004) and *O. niloticus*(Golow and Godzi, 1994).

The previous reports of deltamethrin toxification posed the critical influences on fish physiology. The hypocalcemia, observed in *Heteropneustes fossilis*, a freshwater catfish, after the exposure of deltamethrin for 96 h with a LC_{50} value of 1.86 µg/L, could be the response of impairment of electrolyte influx at the gill (Srivastav et al., 1997). In the long-term exposure of deltamethrin (0.37 µg/L for 28 days), prolactin cells of *H. fossilis* exhibited hyperactivity which is inferred by degranulation and accreted nuclear volume to cope with the hypocalcemia (Srivastav et al., 2010). Further, Srivastav et al. (1997) also stated that the exposure elicited hypophosphatemia due to the redistribution of electrolytes between the intracellular or the extracellular compartments of renal function. In furtherance, it has been represented that the erythropoiesis have been accelerated to avoid from anaemic state tending to increase the production of erythrocytes in a research by Kumar et al. (1999), they pointed to the alterations in morphological and functional structure of renal tubules and liver cells, which the LC_{50} value is recorded as 0.52 µg/L for the same specie, *H. fossilis*, as indicated above. Excess of melano macrophage centers, congestion, odema and lymphocytic infiltrations were observed for liver tissue, whereas hemorrhages in several sites of kidney associated with renal tubule damage in *Clarias lazera* were noticed after acute exposure of 0.5 µg/L for one week (Aziz et al., 2009). Moreover, gill activity and

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heart NA^+ K^+-ATPase were inhibited by deltamethrin exposure whereas the liver total CYP450 and EROD activity were induced in *Ancistrus multispinis* as reported by De Assis et al. (2009). Besides its neurotoxic characteristic, Haverinen and Vornanen (2014; 2016) asserted that deltamethrin is also cardiotoxic to fish and this response could be interpreted with the high affinity of fish Na^+ channels to deltamethrin as in other pyrethroids.The immunosuppressive and oxidative stress inducing potencial of deltamethrin were evidenced in various researches including different fish species and should not be underestimated. The dose of 2 and 4 μg/L of deltamethrin conduced cell-mediated and humoral-mediated immunosupression in rainbow trout which left the fish vulnerable to viral and bacterial disease (Siwicki et al., 2010). Due to the primary sites of deltamethrin absorption, gills are the most susceptible tissues to exposure and it is not puzzling that the reducing levels of oxidative stress indicators in terms of catalase, GST and GPx were obtained from the gills of *Channa punctatus* after 0.75 μg/L of deltamethrin treatment (Sayeed et al., 2003) and this coincided to a series of researches in rainbow trout as performed by Ceyhun et al. (2010a,b).

THs participates in several metabolic functions in terms of development, growth and metamorphosis in fish (Nelson and Habibi, 2009) and presented their activity by binding to *TR*s (genomic pathway) or through a rapid non-genomic signaling pathway (Manchado et al., 2009), nonetheless the action mechanism for fish is not fully understood.*TR*s are members of a large superfamily of nuclear receptors (NR), along with steroid hormones, retinoids and vitamin D (Mortensen et al., 2006). *TR*s have a core role in the genomic regulation of THs (Power et al., 2001), particularly they appear in the early development and metamorphosis in fish (Manchado et al., 2009; Filby et al., 2006) and serve different functions and demonstrate tissue-specific and developmental state-specific expression (Chen et al., 2002; Tu et al., 2016). EDCs, which deltamethrin is regarded as acting a member of this extensive compound group (Mnif et al., 2011)induce to changes, particularly inhibitory ones, in formation and synthesis of THs, affect their bioavailability by binding deiodinases which is responsible for activation or inactivation of THs in peripheral tissues (Kloas and Lutz, 2006). In the present study, *TRα* was up-regulated in comparison to *TRβ* for both liver and white muscle tissue after sublethal exposure of deltamethrin-based formulation, despite the differences were found statistically insignificant. The increased mRNA levels of *TRα* was totally associated with *TR*s subtypes (*TRα* and *TRβ*) as mentioned by Filby et al. (2006). Tu et al. (2016)reported that bifenthrin and λ-cyhalothrin, two synthetic pyrethroid, exposure conduced to remarkably higher mRNA levels of *TRα*gene in zebrafish which is consistent with this study results. Considering these results, exposure of pyrethroids comparatively can be responsible for the disruption of motor activity associated with the alterations of TH levels (Brander et al., 2016) and concurrent increase in calcium values (Kaul et al., 1996).

Conclusion

Overall results of this study, suggest that sublethal exposure of deltamethrin-based insecticide Metrodesis, led to alteration in expression of *TR*s subtypes, specifically *TRα*, independent from tissue-manner, emanating wider potential impacts on the physiological function of fish. Considering the extensive use of this chemical and its high toxicity to aquatic organisms, the specification of risk assessment, particulary endpoints and molecular mechanisms regarding toxicity is a dictate of environmental

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sustainability. Further studies are needed to understand the dose-response relationship between deltamethrin and deltamethrin based formulations and endocrine disruption centering upon non-target organisms as fish.

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BIODIESEL FROM MICROALGAE USING SYNTHESIZED NOVEL ALKALI CATALYST

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Abstract. The aim of the study is to be able to create a solid catalyst that would be sourced from cheap materials, inexpensive to produce, and can comparatively produce high FAME yields in transesterification reactions involving microalgae as lipid source. The impregnation method was used to synthesize the catalysts Li-pumice, K-pumice, and Na-pumice. The synthesis parameters were varied at different solution concentrations to determine which created the best performing catalyst in the transesterification process. Catalyst characterization was evaluated using Hammett indicators, Scanning Electron Microscopy (SEM), Energy-dispersive X-ray Spectroscopy (EDS), and Infrared Spectroscopy (IR). The catalyst was easily separated by filtration and further investigation proved that it can be reused multiple times in transesterification processes. The best performing catalyst was observed to be K-pumice and the optimum concentration of the contact solution in the synthesis was 1.0 N. The transesterification reaction was able to generate an optimum of 77% Fatty Acid Methyl Ester (FAME) yield when process parameters were: reaction temperature = 60 ºC; methanol to oil ratio = 18:1; catalyst load = 10%; and reaction time = 2 hours. The results were able to prove that effective catalyst for biodiesel production can be sourced from cheap materials such as pumice and that the preparation of such can be done in a fast and cost effective manner. It is encouraged that more cheap materials should be investigated for catalyst synthesis that would become ideal for biodiesel production.
Keywords: *bioenergy, pumice, synthesis, transesterification, yield*

Introduction

The spike in the demand of fuel globally and the realization that fossil fuels will be depleted in the near future had caused scientists and opportunists to look for an alternative. For years, researchers have gone back investigating biofuels as the alternative in the exhaustion of fossil fuels (Meher et al., 2006). Not only can biofuels replace the impending loss of fossil fuels, it also has environmental benefits such as a lesser greenhouse gas emissions when compared to fossil fuels. The cultivation of plants as a result of biofuel production also sequestrates carbon dioxide in the atmosphere (Slade and Bauen, 2013).

Biodiesel, a biofuel, is produced by transesterification process were oil is mixed with alcohol in the presence of a catalyst. The oil could be from canola, peanut, corn, animal fat, rapeseed, palm, soybean and waste cooking oil (Singh et al., 2014). Other raw materials that gained importance as potential feedstock for biodiesel production are karanja, rubber plant, tobacco, jatropha, mahua, rice bran, animal fats and more

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recently, microalgae (Borugadda and Goud, 2012). Microalgae is currently considered to be the most feasible alternative since it has a higher photosynthetic yield (3-8%) compared to other plants (less than 1%) that makes them grow faster (1-3 doublings per day) (Slade and Bauen, 2013) (Alam et al., 2012), sequestrate carbon dioxide much better, and accumulate lipid yield of up to 50% (Spolaore et al., 2006). Moreover, microalgae can grow in arid lands, swamps, and saline or fresh water which makes them a non-competitor in land utilization against others crops whether for fuel or food production (Alam et al., 2012). Also, microalgae are a tough species that makes them tolerant severe environmental conditions where losses due to calamities such as floods and storms are negligible when compared to other terrestrial plants (Najafi et al., 2011).

The downside of biodiesel production from microalgae are the cost associated with microalgae cultivation, lipid extraction and oil purification (Raslavičius et al., 2014). To minimize costs associated with algal biodiesel production, certain processes and catalyst were evaluated. The usual catalyst for biodiesel production are homogenous alkali catalyst like NaOH or KOH (Lam et al., 2010). However, there is cost associated with its separation from the final product and full recovery are always a challenge. A few studies have recommended the use of synthesized catalyst but they are either expensive or underperforming (Sharma et al., 2011; Díaz and Borges, 2012). Synthesized catalysts had been made by using metal oxides such as calcium oxide and magnesium oxide (Zabeti et al., 2009), metal complexes (Tariq et al., 2012), and metal hydroxides (Wang et al., 2013). The ease of separation and more cost efficient downstream processes were highlighted in these studies. However, the formation of the three phase system proved to be a huge factor in leading to low FAME yields (Zabeti et al., 2009). This study presents the synthesis of a catalyst that promises to be cheap, easier to prepare and can deliver biodiesel with acceptable methyl ester yields.

Materials and Methods

Materials

Pumice powder was purchased from Sigma Aldrich Chemical Corporation, USA. The microalgae was provided by the Food Protein Research and Development Center (FPRDC) of Texas A & M University (TAMU). The microalgae was in dried colletized form and are labelled to be 95% *Chlorella vulgaris* with 8-10% moisture. Other chemicals and reagents used in the oil extraction, transesterification and analysis of biodiesel were purchased from Sigma Aldrich Chemical Corporation, USA and VWR Chemical Corporation, USA.

Preparation of Catalyst

Pumice powder was used in this study as the support for the synthesis of metal-pumice catalyst. Pumice was initially dried in an oven at 120 ºC for 3 hours after which it was ion exchanged by impregnation method with an alkali solution for 24 hours. The alkali solution varied in terms of concentration and kind. There were three kinds of alkali aqueous solutions used in this study namely KOH, NaOH and LiOH. Concentrations of the solutions also varied from 0.5N to 4.0N. After ionic exchange, the pumice was recovered and dried again in an oven at 120 ºC for another 3 hours. The subsequent product was then kept in a tightly sealed jar ready for evaluation and transesterifcation processes.

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Catalyst Characterization

Pumice and metal-loaded pumice (K-pumice, Li-pumice, and Na-pumice) were characterized using Hammett indicators, X-ray Diffraction (XRD), Scanning Electron Microscopy (SEM) and Infrared Spectroscopy (IR).

The Hammett Indicators measured base strength of the catalysts and its base total amount. The base strength is measured with pH of aqueous solution saturated with catalyst (20g / 50 ml). Base total amount was measured by titrating 0.1N Benzoic Acid in Benzene to the mixture of 1g catalyst sample in 20ml Benzene with 1 ml indicator solution (phenolphthalein in ethyl alcohol).

The SEM and EDS analysis was done with the JEOL JSM-7500F, an ultra-high resolution field emission scanning electron microscope (FE-SEM) equipped with a high brightness conical FE gun and a low aberration conical objective lens. The catalysts samples were loaded into the equipment in one batch. The samples were labelled each prior loading into the equipment. For each analysed sample, at least three areas for were chosen purposely with images taken thereafter ranging from 1 µm to 100 nm. The best image taken from each sample was presented in these paper. The EDS analysis were then done right after the images were taken. Sites in the surface of the samples that were evaluated by the EDS were also purposely chosen.

The IR measurements were performed with the Shimadzu IR Affinity-1 equipment. The KBr pellet technique was used in determining IR spectra of the samples at room temperature. The range was adjusted at 600-4500 cm^{-1}, with 300 scans and 4 cm^{-1} resolution.

Transesterification Reaction

The transesterification experimental runs started with the oil extraction from the milled microalgae. The extraction was done via Soxhlet facility whereby 500g of microalgae was loaded per batch and the extracting solvent was 250ml of n-hexane. The whole extracting procedure was done after about 20 passes which took 6 hours. After the distillate was cooled to room temperature, it was transferred to the rotary evaporator to separate the algal oil from n-hexane. The algal oil was then further purified using the methods described by Tyagi et al. (2012).

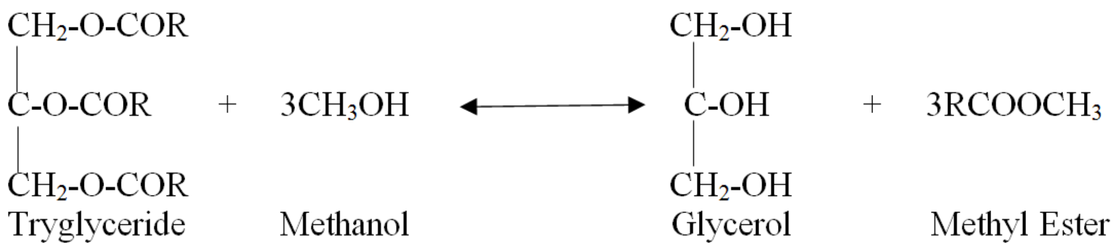

***Figure 1.** The transesterification reaction*

The transesterification reactions (as described in *Figure 1*) were carried out in a 125 ml three-neck glass flask equipped with a stirrer, a water cooling condenser and an electric jacket with thermocouple. Five grams of initially extracted and purified algal oil was placed into the flask and was heated in the range 40-60 ºC while stirring. After the oil has reached the desired temperature, methanol and the catalyst where carefully added to the system. The kind of catalyst (Li-pumice, K-pumice, Na-pumice), amount

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of methanol (methanol to oil ratio 6:1-22:1) and catalyst loading varied in each transesterification procedure (5-20% by weight of algal oil). The reaction time for each transesterification process was also varied from 30 minutes to four hours. After transesterification, the mixture was filtered to separate the catalyst from the liquid. The liquid mixture was then subjected to the rotary evaporator to remove methanol. It was then placed in a separatory funnel and laid to rest for six hours before removing the glycerine that settled at the bottom of the funnel. The remaining liquid, which is presumed to be mostly biodiesel, is washed with hot water (60-80 ºC) to dissolve and separate soap formed during the reaction. The water is removed using again the separatory funnel. The washed biodiesel was then weighed and a sample was collected and loaded to the Shimadzu GC-2010 with Zebron ZB-5MS column for qualitative analysis. Meanwhile, the separated catalyst from the transesterification reaction was washed with methanol, dried and calcined for 12 hours in a temperature of 300 ºC. It was again regenerated by ion exchanging in a 1.0 N alkali aqueous solution and placed in a 120 ºC for 3 hours. The regenerated catalyst was then left to cool in a desiccant where it was later transferred in a cool dry jar for storage.

Analysis

The fatty acid methyl ester (FAME) yield of biodiesel was calculated by its weight in the crude biodiesel in relation to the weight of oil extracted from the algal biomass. The composition of FAME contained in the washed biodiesel was analysed by GC-MS. The biodiesel samples collected were dissolved in dichloromethane in the ratio 1:4 and loaded into the GC trey. The carrier gas used was helium and the injection volume of the sample was set at 1.0 µl at an injection temperature of 180 ºC. The temperature program of the GC-MS was initially set at 50 ºC held for 5 min, increased to 250 ºC at 5 ºC /min, and held at 250 ºC for 10 min. The relative weight composition of the sample was determined using methyl stearate as the standard. Data gathered from all experiments were carefully recorded and were done in triplicate.

Results and Discussion

Catalyst Characterization

The results of Hammett Basicity Test (*Table 1*) suggested that considerable difference in base total amount can be observed in between catalyst that were impregnated in 0.5N solutions and 1.0N solutions. As concentration of contact solution increases, so is the base total amount but of negligible margins for solutions with concentrations more than 1.0 N. Base strength is almost unanimously the same for contact solutions 1.0N and above. K-pumice generally is more basic and has higher number of basic sites while Li-pumice has the least.

Table 1*. Base Strength and Base Total Amount of Synthesized Catalysts*

Catalyst	Contact Solution (N)	Base Strength	Base total amount (m mol /g)
Li- pumice	4.0	12.1	50
	2.0	12.1	47
	1.0	11.7	33
	0.5	11.3	2

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Na-pumice	4.0	12.4	56
	2.0	12.4	55
	1.0	12.2	47
	0.5	11.9	7
K-pumice	4.0	12.6	62
	2.0	12.6	60
	1.0	12.5	55
	0.5	12.1	12

The EDS values (*Table 2*) are relative weight percentages of the elements found in the surface of the catalysts evaluated. The catalysts presented in the table are Na-pumice and K-pumice contacted with a 1.0N solution. The Li-pumice was not included upon EDS evaluation since the equipment could not detect spectra of elements lighter than carbon which included lithium. It was also assumed that the same values will transpire for catalysts that were contacted with solutions greater than 1.0N since their base strength and base total amount are relatively similar to those catalysts contacted with 1.0N solution. In retrospect, the value of base total amount indicates the quantity of metal impregnated in the catalyst support (Galadima and Muraza, 2014).

Table 2. *Relative weight percent composition of pumice and metal impregnated pumice*

Element	Blank	Na	K
C	15	14.5	14.5
O	59	58	58
Si	4	4	4
Al	18	17.5	17.5
K	1.5	1.5	3.5
Na	1	3	1
Others	1.5	1.5	1.5
Total	100	100	100

As noted in *Table 2*, the blank pumice is predominantly composed of silicon, aluminum, carbon and oxygen being it a volcanic rock that is mostly silica and alumina. It initially has 1% sodium and 1.5% potassium. By impregnating it with a metal hydroxide solution, the relative weight percentage of the element is increased by 2%, thus the increase in the basic sites of the synthesized catalyst. On this presumption, the Li-pumice (with 1.0N or above contact solution) may likely also to have 2% lithium in its over-all weight composition. The elements that listed as others are magnesium, calcium, palladium, iron, nickel and nitrogen.

The FTIR readings (*Figure 2*) of the selected catalysts displayed there absorbance from 600 to 4000 cm^{-1}. The catalysts that were evaluated was limited to 6 since the objective is to demonstrate the difference in the infrared spectrum of absorbance in pure pumice, pumice impregnated with different metals, and pumice impregnated with different concentrations of alkali aqueous solution.

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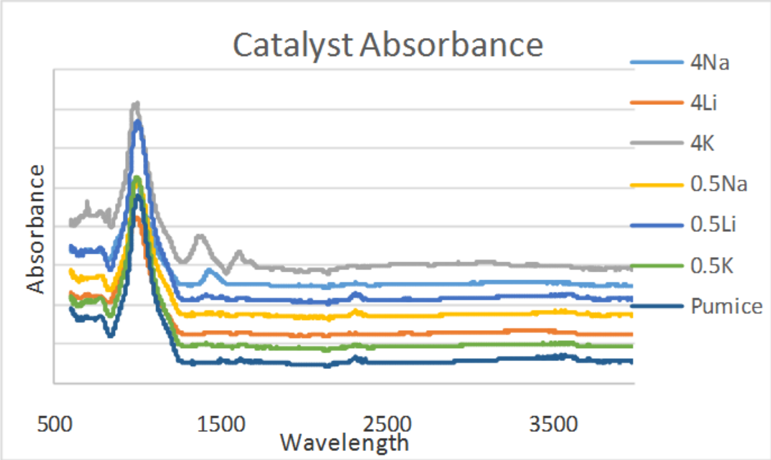

***Figure 2.** Catalyst absorbance of pumice and selected synthesized catalysts*

The peaks at 600 and 800 cm−1 in the FTIR spectrum of the pumice and metal loaded pumice may have resulted from the Si–O bending strength vibrations of the amorphous quartz which constituted the structure while the strong peak around 1000 cm^{-1} may have resulted from Si–O stretching vibrations (Selli and Forni, 1999). Pumice is predominantly composed of SiO_2 (70%) and Al_2O_3 (15%). The noticeable difference on the FTIR spectrum in the strongest reactive catalyst (K-pumice contacted with 1.0N KOH solution) and the weaker ones are the peaks detected between 1300 and 1600 cm^{-1}. According to E. Selli et al. (Selli and Forni, 1999), the peak can be attributed to the Metal–O bond structure vibration that was formed in the surface of the pumice during the activation of the catalyst. Such peaks may not have been present in pumice that had been contacted in lower concentration solutions since the metals (K, Li, Na) may have been embedded in the pores and not enough ions was able to have been adsorbed in the surface. The peaks are also noticeable but not as quite pronounced in the Na-loaded pumice (with 4.0N as contact solution). The peak that could be observed around 2400 cm^{-1} for all the catalyst sampled pointed out the -OH stretching vibrations of the water (moisture) which was adsorbed by the sample from the outside environment (Selli and Forni, 1999).

The SEM images (*Figure 3*) were taken out of the selected three kinds of catalyst and the blank pumice. All images were taken to almost the same resolution and magnification for efficient and proper comparison (see scale = 1μm). It can be noted that the pure pumice has mostly rough surfaces and orifices which are ideal for metal catalyst impregnation.

Shown also in an isolated case is a smooth surface with the absence of anything attached to it. For pumice impregnated with metals, the surfaces clearly has some particles that can be observed. The images though would be a bit imprecise to judge the differences on the appearance of the particles attached on the surfaces of pumice support with different metal impregnations. The images that were taken were just plainly made to observe what is in the surface of the impregnated pumice and to see how it is synchronized. Eminent in the images taken are the wavy-like material on the surface of K-pumice and the long and crystalline in shape (resembling fine broken glasses) in the surface of Na-pumice.

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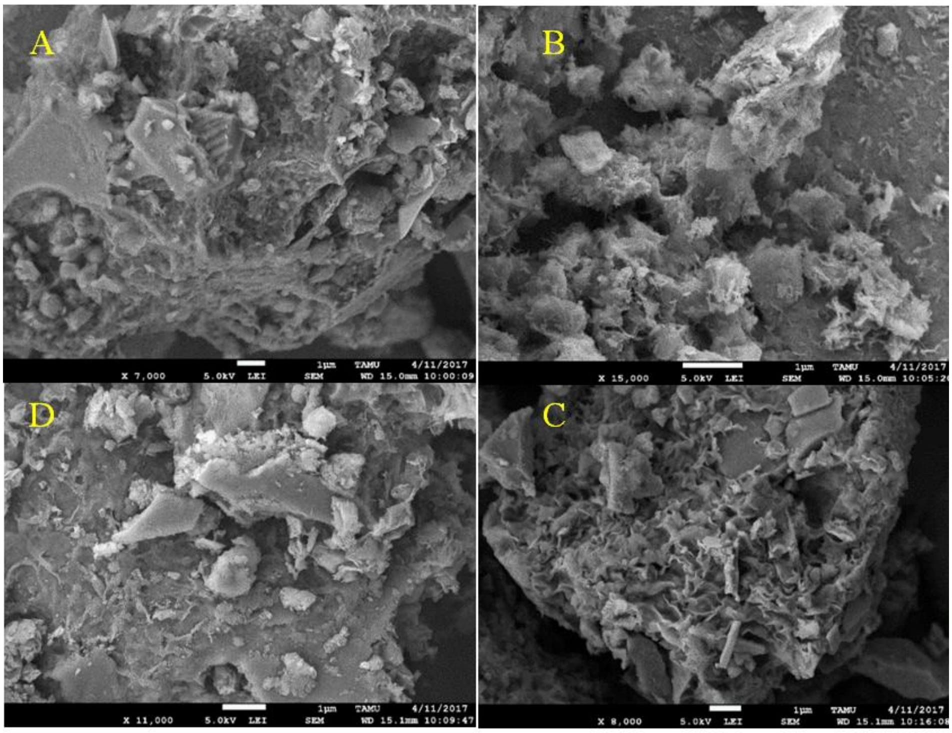

Figure 3. *SEM images of pumice and metl-impregnated pumice. Clockwise from upper left: a) Pure pumice powder; b)Na-pumice; c) K-pumice ; d) Li-pumice*

Transesterification Process

The process parameter that was firstly investigated was the concentration of the contact solution. With the other variables fixed at 20% catalyst load, 18:1 methanol oil ratio, reaction temperature at 60 ºC, and reaction time equal to 2 hours, it was observed that K-pumice and Na-pumice with contact solutions 1.0N to 4.0N gave the best FAME yields (see *Figure 4a*). K-pumice had peaked at 77% FAME yield when the concentration of contact solution is 1.0N and the same yield have been recovered when the concentration of contact solution had been increased up to 4.0N. Na-pumice also had about 75% FAME yield at the same process conditions and just slightly increased to 76% when contact solution is increased up to 4.0N. Li-pumice had about 5% lower FAME yield as compared to the other two catalysts ion exchanged in the same concentration of contact solution.

During shorter reaction times, it is observed that K-pumice had a better FAME yield than Na-pumice and that the FAME yield gap between K-pumice and Li-pumice becomes larger. This phenomena maybe attributed to the difference in solubility of the three metals in methyl alcohol, with potassium to be the most soluble and lithium to be the least (Gryglewicz, 2000). Nevertheless, it is observed that the optimum contact solution for all catalyst is 1.0N. It is thru this concentration where the possible highest FAME yield was achieved for K-pumice and the curves plotted in *Figure 4* for the other catalysts suggested it to be the most optimum.

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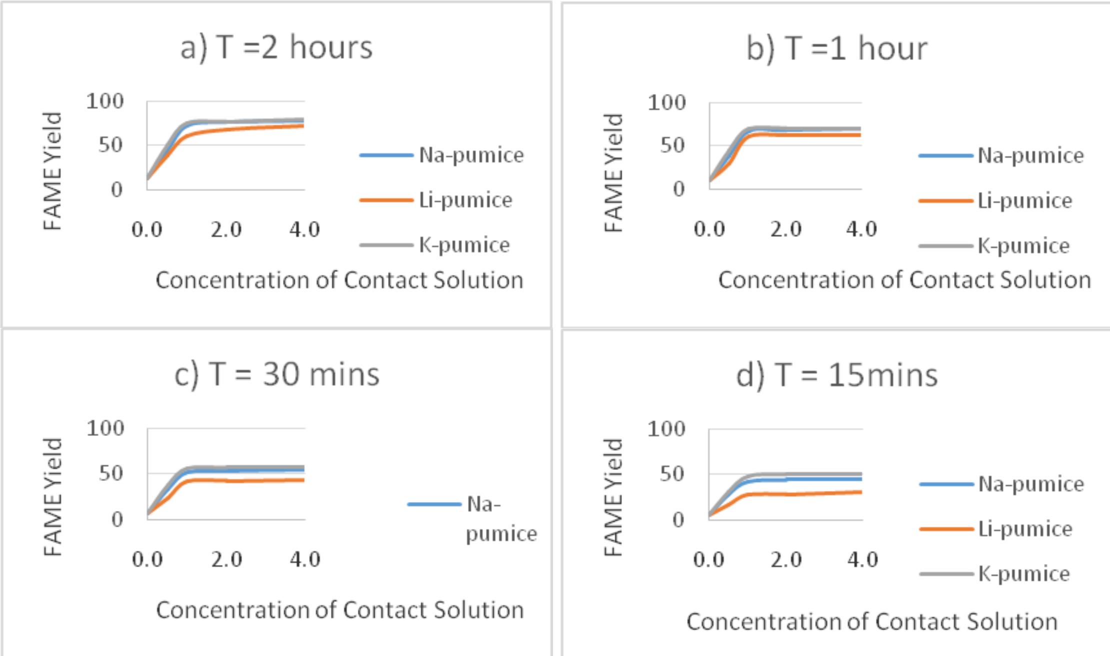

Figure 4. *Process performance in terms of FAME yield of catalysts impregnated with different concentrations of contact solution at different reaction temperatures.*

When the investigation was focused upon the optimum reaction time (*Figure 5*), the other process parameters were fixed at methanol to oil ratio at 18:1, reaction temperature equal to 60 ºC, catalyst load at 20% and the catalysts used were those contacted with 1.0N solution. Both Na-pumice and K-pumice achieved the optimum FAME yield (75-77%) after 2 hours of transesterification while Li-pumice still has an improved FAME yield after 4 hours. The best yield however that was acquired in the setup was that of K-pumice just after 2 hours of transesterification.

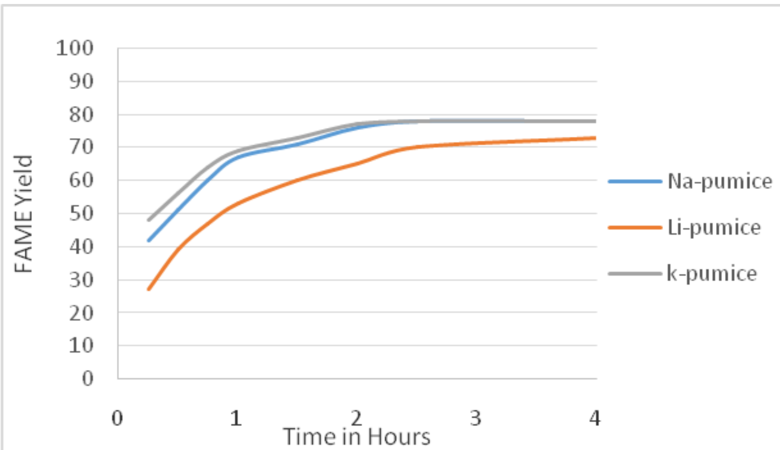

Figure 5. *Influence of reaction time on the FAME yield using different types of catalysts*

In the investigation of the optimum catalyst load (*Figure 6*), methanol to oil ratio (*Figure 7*), and reaction temperature (*Figure 8*), the catalysts used were limited to that

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contacted with 1.0N solution. The other synthesized catalysts were not used in these experiments since it was proven earlier that the optimum concentration of contact solution is 1.0N. Also, the reaction time in these investigations were set at 2 hours.

The optimum catalyst load that was observed during these transesterification reactions was 10% for all the kinds of catalyst (see *Figure 6*). As with the data presented earlier, it had been noted that Li-pumice performed the least while K-pumice slightly fared better than Na-pumice. The methanol to oil ratio and reaction temperatures on these catalyst load optimization were set at 18:1 and 60 ºC, respectively.

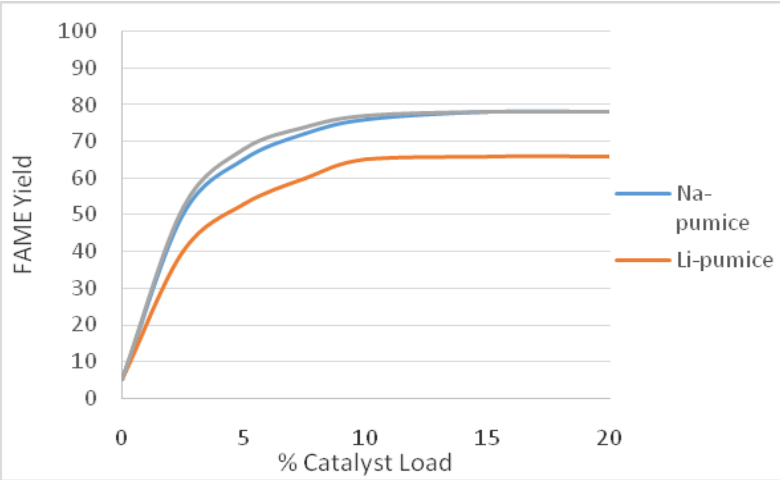

Figure 6. *Influence of catalyst loading on the FAME yield using different types of catalyst*

As far as the analysis on the methanol to oil ratio was concerned, the experiments showed that the optimum value is 18:1 (see *Figure 7*). Increasing the amount of methanol beyond that does not seem to increase the FAME yield of the reaction. Even though the optimum ratio in transesterification experiments is 6:1 when the catalyst is homogenous and alkali in nature, the same ratio using the catalyst being used in these experiments can only give a maximum of 35% FAME yield. It is most likely because of the three-phase system in the reaction that more alcohol should be introduced in order to hasten the reaction forward.

Finally, the investigation on the optimum reaction temperature only settled the long standing observation that the best reaction temperature of a transesterification process is the temperature just slightly below the boiling point of the reacting alcohol (Bharathiraja et al., 2014). Since methyl alcohol was used in these transesterification proceedings with a boiling point around 64.7ºC at atmospheric conditions, the optimum reaction temperature obtained in these experiments were 60ºC (see *Figure 8*). Having the reaction temperature above the boiling point of the alcohol can make it to evaporate, lessening the interaction of oil and alcohol for them to produce biodiesel.

The reuse of the synthesized catalyst in this study was also scrutinized. It is very important that the catalyst should be able to prove itself for reuse in order to justify its use economically. The experiments reused the catalyst that were earlier used in prior transesterification experiments. The investigation covered the reuse of the catalysts that were regenerated and those that were not. Regeneration is the term used in this study whereby the catalyst is being washed by methanol, dried, calcined and impregnated

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again in 1.0N contact solution (NaOH, KOH, or LiOH). Those that were not regenerated was just dried, calcined and used again on the next transesterification process. The process conditions in the reusability transesterification processes were the optimum as based on the earlier experiments.

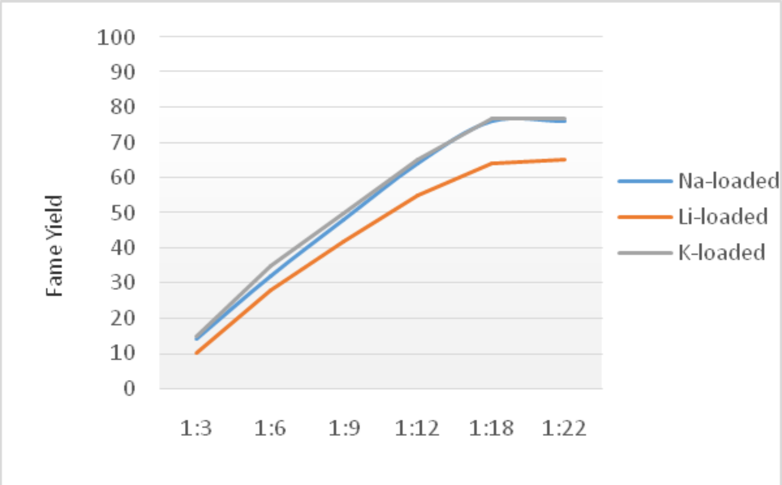

Figure 7. *Influence of methanol to oil ratio on the FAME yield using different types of catalysts*

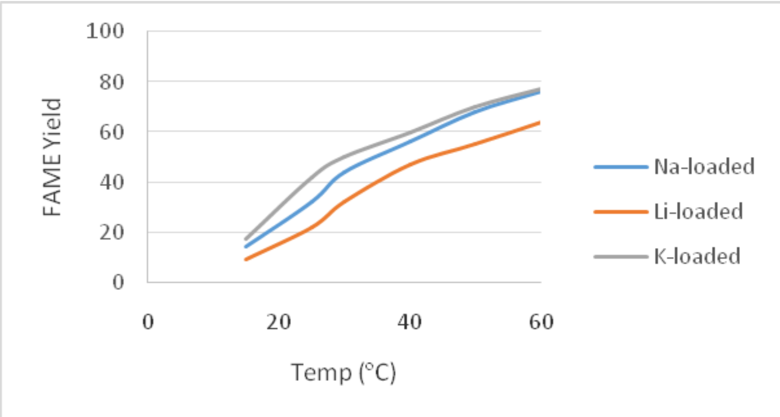

Figure 8. *Influence of reaction temperature on the FAME yield using different types of catalysts*

The reusability experiments discovered that without regeneration, the most effective number of reuse that the synthesized catalysts could do is just up to three uses (see *Figure 9a*). If it would be regenerated, the catalyst can be reused multiple times over without affecting its prior performance (*Figure 9b*). It is worth noting that blank pumice can be able to produce biodiesel even at a lower yield. It is most probable that the surface of the material has catalytic properties that can support transesterification reactions even if it was not impregnated at all by an alkali solution. Also, the data in *Figure 8b* showed the resemblance of the curves by the three kinds of catalyst even when they are at different levels. It could indicate that the reusability can particularly be associated with the physical nature of the catalyst support which in this case is pumice.

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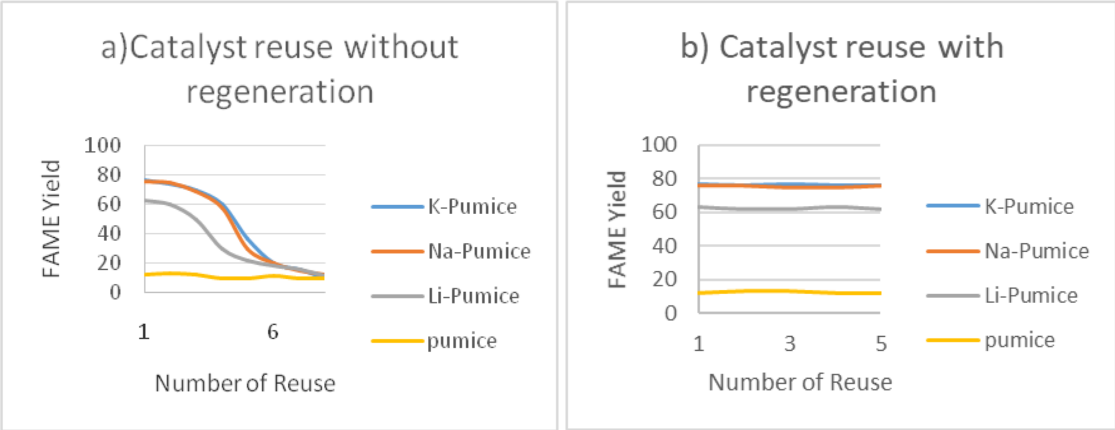

Figure 9. *Reusability of the catalysts*

Conclusion

The synthesized catalyst that was made by ion exchanging an alkali solution with pumice powder proved to be one of the better alternatives to the pre-existing catalysts in the production of biodiesel. The cheap cost of the catalyst support which is pumice, and the ease of preparation of the catalyst make it viable in transesterification processes. It was easily separated by filtration and was proven to be effective in reuse with regeneration. The best performing catalyst as far as FAME yield is concerned was K-pumice, followed by Na-pumice and then Li-pumice. The optimum contact solution in the synthesis of the catalysts were 1.0N. The K-pumice catalyst was able to generate FAME yield of 77% from microalgae. The optimum process conditions to guarantee maximum FAME yield when using K-pumice as the catalyst are: reaction time = 2 hours; reaction temperature = 60; methanol to oil ratio = 18:1 and catalyst load = 10%. The current FAME yield could still be improved by tapping to current technologies that are being used in transesterification reactions. It is strongly recommended that ultrasound and microwave technologies be incorporated to the current set-up since it may affect positively the yield of the resulting biodiesel. Also, it would be interesting if the synthesized catalyst in this study be tried for biodiesel production using other lipid source such as animal fat, waste vegetable oil, or others sources that are considered to be waste. Further investigation in catalyst synthesis using cheap materials not yet tried for biodiesel production will be encouraged.

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COMBINATION OF WASTE-HEAT-RECOVERY HEAT PUMP AND AUXILIARY SOLAR-ENERGY HEAT SUPPLY PRIORITY FOR TOBACCO CURING

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Abstract. Extensive management of tobacco curing facilities can lead to fuel waste and environmental pollution. In this study, a new integrated equipment controlled by a microcomputer is proposed and built, including a flat panel solar-collector heating system located on the unoccupied flat rooftops of a bulk curing barn, a heat-pump heating system, and a heat-pump-type dehumidification system. Test results show that from 10:00 am to 3:00 pm, under sunny or partly cloudy weather during tobacco season, the air temperature in the flat-panel solar collector exceeds 68 °C, which is higher than the highest dry-bulb temperature required for tobacco curing. Compared with an existing air-source heat pump heating system in the barn, a curing cost saving of 21% per kilogram of dry tobacco leaves is achieved. Because of waste-heat recovery for recycling and solar energy heating, the operating time of the new device heating compressor is approximately 70.6 h during a curing time of 142 h, which is less than the control compressor, i.e., 115.8 h. The heat pump dehumidification system controls the difference between the actual wet-bulb temperature and target wet-bulb temperature of the barn to be within 0.5 °C.
Keywords: *hot air drying, dehumidifier, precision control, dry-bulb and wet-bulb temperature, bulk curing barn*

Introduction

Flue-cured tobacco is dried by fire in a process generated inside a furnace and the heat is transferred through the flue pipes laid in a strategic position inside an airtight barn where the leaves are hung (Truong et al., 2016). The large-scale use of coal fuel for tobacco curing can result in environmental degradation (Li et al., 2017; Wang et al., 2015). In recent years, with serious haze weather conditions occurring frequently in North China and more than 30% (2.3 million ton) of flue-cured tobacco leaves produced in China, air pollution caused by coal burning and heating used for conventional tobacco curing has received significant attention (Liu et al., 2016; Mana and Fatt, 2017). The government attaches considerable importance to the use of clean energy for tobacco curing and emission reduction (Xiao et al., 2015).

Literature Review

Solar energy is an abundant, renewable, and sustainable energy source that has attracted several eminent researchers globally to work in the field of solar energy applications for flue-cured tobacco leaves (Kumar et al., 2016; Patil and Gawande,

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2016). From 1974 to 1986, Huang and Bowers Jr. (1986) developed two types of solar greenhouse designs namely the load supporting wall type and the shell type for tobacco curing. Janjai et al. (1986) investigated the technical feasibility of using solar energy for flue curing of tobacco in a bulk barn. Subsequently, Indian tobacco operators built a solar barn in Lajiawang, Delhi, which could save 43%–45% fuel (Subramaniam et al., 1998; Randrianarison and Ashraf, 2017). China is one of the first countries to use solar energy for tobacco curing. With the extensive application of bulk curing barns since 2008, a few researchers built a solar heating system on the unoccupied roof of a barn that was used only in the tobacco yellowing period (Lu et al., 2011). Limited by monolithic computer technology, the early solar energy system used for flue curing of tobacco was relatively simple and could not be controlled accurately.

Heat pump technology was first introduced in Germany in 1930, and it was rapidly applied and promoted in all fields (Carl, 1933). Owing to its significant advantages, such as energy-saving and reduced emissions, heat pump drying became widely used in the industry (Bao and Wang, 2016). Gong and Pan (2003) were the first to use the heat pump technology to provide independent heat for tobacco curing, and they built numerous barns that were heated using a heat pump in a few tobacco planting areas in China. Heat pump dehumidification led to recovery of waste heat for recycling (Ahn et al., 2016; Chantoiseau et al., 2016). A heat-pump dehumidifier was used by Maw et al. (2004) in 1999 for the first time for tobacco curing, which was only applied during the lamina and stem drying phases (Wu et al., 2017).

Based on the above literature, a device that combined a waste-heat-recovery heat pump with an auxiliary solar-energy heat supply priority for tobacco curing was developed for use in a single bulk curing barn. Through precise control of curing process parameters, the objective was using the adequate solar energy during the season of tobacco plant harvest and the recycling of heat from the high temperature moist air during the tobacco curing process to cure tobacco leaves.

Materials and methods

Equipment structure

The integrated energy-heating equipment consists of a heating structure with a distinct appearance (*Figure 1*), which includes a solar air heating system, a heat pump heating system, a heat pump dehumidification system, a microcomputer, and other components. The equipment power supply is 380 V. In China, the basic structure and specifications of a bulk curing barn were established by the State Tobacco Monopoly Administration on April 18, 2009 (hereinafter referred to as barn no. 418) (TSBCN, 2009). The overall size of the barn was 11440 × 2700 × 3500 mm (length × width × height).

The solar air heating system is located on the unoccupied flat rooftops of a bulk curing barn, which is comprised of a flat-panel solar collector (1), a solar hot air chamber (SHAC) (2), an outlet duct for lower temperature air (3), an intake duct for higher temperature air (4), a centrifugal fan (11), etc. Air is the energy carrier medium in the solar air heating system. The centrifugal fan interactively drives airflow between SHAC (2) and the heating chamber to utilize solar energy. It is hermetically connected to the end of the outlet duct for lower temperature air (3). The flat-panel solar arrays are installed facing south and oriented at an angle of 6° to the horizontal. Each barn consists of eight flat-panel solar-collector arrays with dimensions of 3200 × 1200 × 60 mm (length × width × height), which are connected in series through an insulation board and

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airflow pipeline. As a result, one barn with eight arrays forms a solar energy collecting area of 28.8 m^2. Special heat insulation is provided on the roof of the SHAC (2) using a double-glazed organic transparent glass with a thickness of 4 mm and a black multilayer absorbing metal net (23) that is suspended below the glass layer. On the outlet duct for lower temperature air (3), air outlets (22) for different sections have circular holes with different diameters depending on the wind pressure generated by the centrifugal fan (11). Each SHAC is connected in series through the outlet duct for lower temperature air (3) and the intake duct for higher temperature air (4) (*Figure 2*).

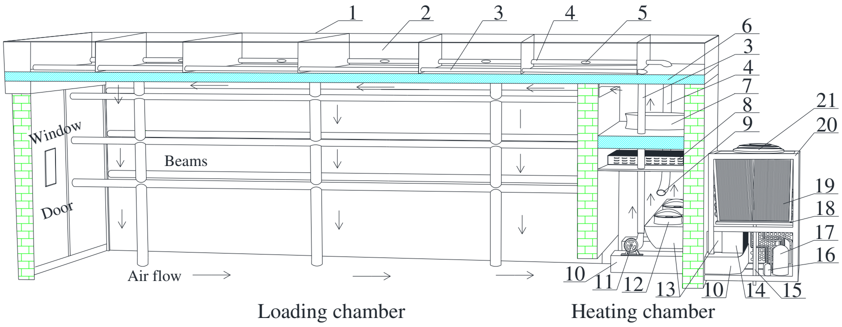

Figure 1. *Structure of the integrated energy-heating equipment.*

Note: 1. Flat-panel solar collector, 2. Solar hot air chamber (SHAC), 3. Outlet duct for lower temperature air, 4. Intake duct for higher temperature air, 5. Hot air inlet, 6. Roof of bulk curing barn, 7. Circulating fan, 8. Reflux condenser, 9. Pipeline exit for solar hot air after heating, 10. Cabinet for moist air entry from the loading chamber, 11. Centrifugal fan, 12. Fan I with a spring cap, 13. Cabinet for dry air outlet after dehumidification, 14. Evaporator I for dehumidification, 15. Pipeline for directing flowing water outside, 16. Compressor I for dehumidification, 17. Compressor II for heating, 18. Channel for flowing water, 19. Evaporator II for heating, 20. Outdoor equipment frame, 21. Fan II for external heat exchange.

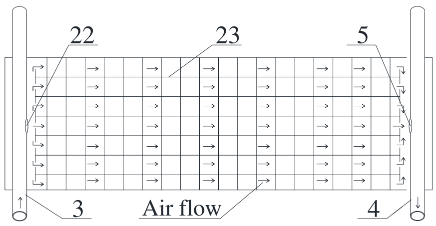

Figure 2. *Internal structure of SHAC.*

Note: 22. Air outlet, 23. Black multilayer absorbing metal net.

In the reflux condenser (8), there are two sub-condensers for heat pump heating and heat pump dehumidification drying, which share a heat pipe fin and the structural frame of the equipment. However, the condensate pipes of the sub-condensers are separated. The reflux condenser (8), compressor I for dehumidification (16), evaporator I for dehumidification (14), two cabinets for moist air entry from the loading chamber (10), two cabinets for dry air outlet after dehumidification (13), and two fans I with spring caps (12) constitute the waste heat recovery recycling system of the heat pump dehumidification system. The dehumidification system is operated in a closed loop, in which the air inside the barn is dehumidified and re-circulated. During dehumidification, heat is generated and accumulated in the barn. In this manner, higher internal

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temperature and lower relative humidity are achieved, as compared to the case in which fresh ambient air is used and higher temperature air with higher relative humidity is discharged. Evaporator I for dehumidification (14) is the connection point between the two cabinets for dry air outlet after dehumidification (13) and the two fans I with spring caps (12). Compressor I for dehumidification (16) is composed of two single 2.94-kW·h compressors (Emerson Climate Technologies, the same as below). When tobacco leaves in the barn require dehumidification during the curing process, the two fans I with spring caps (12), which are controlled by the microcomputer, start working. Moreover, the caps of the fans open under wind pressure and cause moist air, which is flowing from the loading chamber to the heating chamber, to flow into two cabinets for moist air entry from the loading chamber (10). Moist air is dehumidified by evaporator I for dehumidification (14), and it returns to the heating chamber through the cabinet for dry air outlet after dehumidification (13). Subsequently, the microcomputer stops the two fans I with spring caps (12), and their caps fall down under gravity.

The heat pump heating system is composed of a reflux condenser (8), a pipeline for directing flowing water outside (15), compressor II for heating (17), a channel for flowing water (18), evaporator II for heating (19), fan II (21), etc. Compressor II for heating (17) is composed of two single 2.94-kW·h compressors and two single 4.42-kW·h compressors.

To facilitate the operation of the integrated energy-heating equipment and precisely control tobacco curing, the microcomputer is designed based on curing requirements. The electronic control valve for throttling service is adopted to realize efficient heating and dehumidification of the heat pump.

Working principle

The working principle of the integrated energy-heating equipment is shown in *Figure 3*. The equipment has three independent and coordinated systems for providing heat. Solar energy provides auxiliary heating energy to preferentially assist the heating for tobacco curing. Moreover, energy from the ambient atmosphere, which is independent of the heat pump, primarily provides heat for tobacco curing. The heat pump dehumidifies moist air and recovers waste heat for tobacco curing. The conversion of energy in the three heating cycles is accomplished using the microcomputer operated by a tobacco curing technician.

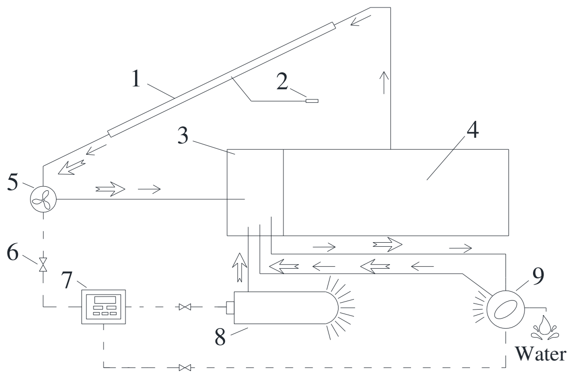

Figure 3. *Schematic of the system's operating principle.*

Note: 1. SHAC, 2. Valve for air pressure relief, 3. Heating chamber, 4. Loading chamber, 5. Centrifugal fan, 6. Control electronic signal, 7. Microcomputer, 8. Air source heat pump heating, 9. Heat pump dehumidification. Note, "⇐" Heat flow direction; "←" Hot air flow direction.

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The heat supply mode is controlled by the microcomputer as follows: 1) The difference between the actual dry-bulb temperature in the curing barn and the target dry-bulb temperature set by the microcomputer is -0.5–0.5 °C. In this case, if the air temperature in the SHAC is more than 10 °C higher than the target dry-bulb temperature, hot air heated through solar heating is driven preferentially by the centrifugal fan through wind pressure to the heating chamber. Then, it is transported by the circulating fan to the loading chamber. Moreover, if the temperature of the SHAC is not more than 10 °C higher than the target temperature, the solar air heating system stops providing heat to the heating chamber, and the heat pump heating system starts working and absorbs heat for tobacco curing from the ambient atmosphere. 2) The actual dry-bulb temperature in the curing barn is lower than the target dry-bulb temperature set by the microcomputer by 0.5 °C. In this case, if the air temperature in the SHAC is more than 10 °C higher than the target dry-bulb temperature, the solar hot air system and heat pump heating system provide heat for tobacco curing. Furthermore, if the air temperature in the SHAC is not more than 10 °C above the target dry-bulb temperature, only the heat pump heating system works. 3) The actual dry-bulb temperature in the curing barn is higher than the target dry-bulb temperature set by the microcomputer by 0.5 °C. In this case, the solar hot air system and the heat pump heating system stop working simultaneously.

According to the requirements of tobacco curing, the heat pump dehumidification system, i.e., evaporator I, converts the excessive water in the high humidity air of the barn into liquid water, while the heat in moist air is recovered for recycling through the reflux condenser in the heating chamber.

To maintain the high efficiency of the heat pump dehumidification system, the speed of fan I is controlled by a variable frequency power, and it is changed at the wet-bulb temperature points from 38–68 °C. The variable speed of fan I can control the ventilation flow between the cabinets for the entry of moist air from the loading chamber and the cabinets for dry air outlet after dehumidification, which could reduce the frost on evaporator I for dehumidification and condense a significant amount of water in the liquid state.

System operation test

The experiment was conducted in the experimental field of the Xuchang branch campus of Henan Agricultural University in 2015 and 2016 (N34°09，E113°49). The curing season typically lasts for 7–9 months, and it is primarily sunny with few rainy days.

Experimental treatment

Three single bulk curing barns (no. 418) with air descent were employed. Barn A was installed with the integrated energy-heating equipment. To facilitate experimental comparison, the equipment in barn B was a conventional heat pump that provided independent heating, and that in barn C was a coal burning furnace, which is widely used locally. Only the heating equipment was different between barns B and C, which had a conventional ventilation-based dehumidifying pattern in which fresh ambient air enters and high temperature moist air is discharged. The mature upper leaves of flue-cured tobacco of cultivar Zhongyan 100, with a uniform quality, were picked simultaneously and hung using the same type of clips used to hang tobacco. The error in the weight of the loaded green tobacco in barns A, B, and C was controlled to be ±50 kg.

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The tobacco curing technology was in accordance with the curing process used in local bulk curing barns.

Basic performance test

When the three single barns started to cure tobacco simultaneously, the target dry-bulb temperature and target wet-bulb temperature (both set by the microcomputer), the air temperature in the SHAC, and the actual wet-bulb temperature in the loading chamber of barn A, were recorded at 1 h intervals from 6:00 am to 8:00 pm.

Working characteristics of system components

Intelligent electric parameter test instruments (Zurui, EPM6600-T) were employed to detect the electric energy consumption and operating time of the circulating fan, compressor I for dehumidification, compressor II for heating, centrifugal fan, fan I with a spring cap, and fan II for external heat exchange. The curing time was recorded, and the moisture lost from tobacco after curing was calculated by measuring the weights of green tobacco and dried tobacco after curing.

Grades and grade indices of cured tobacco

Fifty representative clips of cured tobacco were selected per batch per barn from the same position. Grades were assigned to the selected samples of tobacco by the graders of Tobacco Henan Industrial Company Limited. Grading was performed based on the indices of quality of appearance, which include proportions of superior tobacco, orange tobacco, and lemon tobacco, and whether tobacco leaves are green or black (GB2635-92, 1992).

Results

Heating coincidence degree between the solar energy provided and the tobacco curing required

Figure 4 shows the difference between the heat provided by solar energy and that required for tobacco curing in terms of the target dry-bulb and SHAC temperatures.

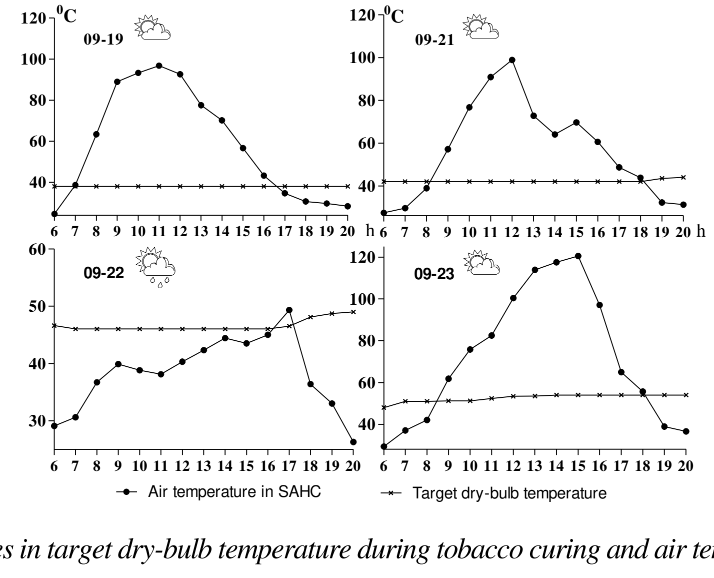

Figure 4. *Changes in target dry-bulb temperature during tobacco curing and air temperature in SHAC.*

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The flat-panel solar collector receives solar energy radiation, and the air temperature in the SHAC rises and fluctuates between ambient temperatures and 120.5 °C. Except in the case of rainy and cloudy weather on 22 September, the air temperature in the SHAC is higher than the target dry-bulb temperature set by the microcomputer from 9:00 am to 5:00 pm, which shows that solar energy could provide heat for tobacco curing. From 10:00 am to 3:00 pm, solar radiation is the most abundant, and the air temperature in the SHAC is considerably higher than the highest temperature (68 °C is the highest dry-bulb temperature required for tobacco curing), which could provide heat during the entire tobacco curing process.

Effect of heat pump controlled dehumidification on tobacco curing

Figure 5 shows effect of heat pump controlled dehumidification on tobacco curing. According to the requirements of tobacco curing, when the wet-bulb temperature is required to remain constant, the actual wet-bulb temperature fluctuates consistently around the target wet-bulb temperature at regular intervals, such as on September 21 and 22. When the wet-bulb temperature is required to rise in the loading chamber, the actual wet-bulb temperature increases, remaining close to the target wet-bulb temperature, such as on September 23. Thus, based on the requirements of tobacco curing, the actual wet-bulb temperature could be controlled by the heat pump dehumidification system to be within ± 0.5 °C of the target wet-bulb temperature.

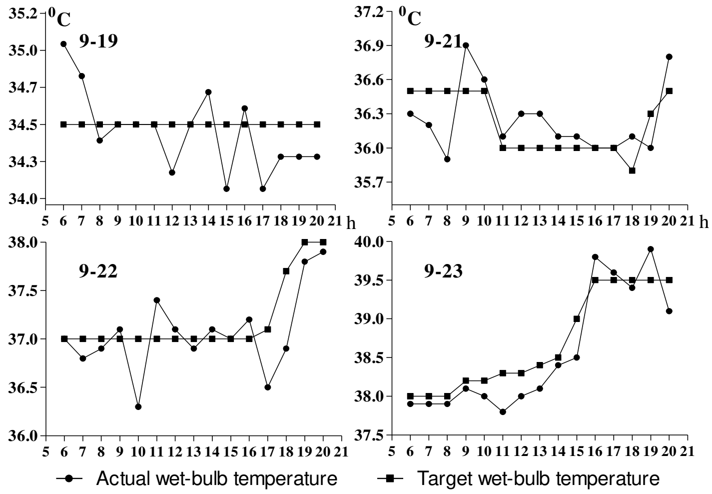

Figure 5. *Change in actual wet-bulb temperature and target wet-bulb temperature.*

Operating characteristics of equipment components

The test results (*Table 1* and *Table 2*) show that the operating time of the heat pump heating system (70.6 h) in barn A is considerably lower than that of the compressor for heating in barn B (115.8 h). The total operating times for barns A and B are 142 h and 141 h, respectively. The heat pump dehumidification system in barn A consumes 347.4 kW power to remove 3815 kg moisture from the tobacco leaves after curing, which is equivalent to 1 kW power for dehumidifying 10.98 kg water from moist air. The curing costs per barn for barns B and C are close to each other; however, they are higher than that for barn A. For the same heat pump heating for tobacco curing, barn A saves 21%

more curing cost than barn B. This suggests that a large amount of heat is lost from barns B and C during the curing process through discharge of hot moist air. With the closed loop operation of the heat pump dehumidification system in barn A, the air from the barn was dehumidified and recirculated, thus attaining a higher internal air temperature and lower relative humidity than is possible by taking in fresh air. Combined with solar energy assisted heating, this could reduce the cost of tobacco curing.

Table 1. *Comparison of equipment operation between barns A, B, and C.*

		Operating hours (h)	**Working power (kW·h)**	**Consumed power (kW)**	**Ratio of energy consumption (%)**	**Energy consumption (Yuan/barn)**	**Curing cost (Yuan/kg)**
Barn A	Compressor I for dehumidification	96.5	3–5	347.4	30.35	744.11	1.03
	Compressor II for heating	70.6	7–13	451.84	39.47		
	Circulating fan	143	1.5–2.2	191	16.68		
	Fan I with the spring cap	70	0.8	79.8	6.97		
	Fan II for external heat exchange	74.6	0.4	21.84	1.91		
	Centrifugal fan	66	0.8	52.8	4.62		
Barn B	Compressor for heating	115.8	8–15	1134.84	85.66	861.15	1.25
	Centrifugal fan	142	1.5–2.2	190	14.34		
Barn C	Coal burning	-	-[a]	-	82.54	810.31	1.24
	Fan for help fuel burning	90	0.13	11.7	0.94		
	Centrifugal fan	153	1.5–2.2	206	16.52		

Note：[a] =1393 kg. The local coal price was 480 Yuan/t in 2015, 2016. The price of electricity was 0.65 Yuan/kW. "-" blank data.

Table 2. *Statistics of the basic curing conditions for barns A, B, and C.*

	Curing time (h)	**Weight of green tobacco (kg)**	**Weight of dried tobacco after curing (kg)**	**Moisture lost from tobacco after curing (kg)**
Barn A	143	4484	669	3815
Barn B	142	4468	670	3798
Barn C	154	4438	632	3806

Tobacco quality after curing

Table 3 shows the appearance quality of flue-cured tobacco after curing. The tobacco appearance quality for barns A and barn B is better than that for barn C, which is

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primarily reflected by a higher orange-yellow tobacco proportion, less tobacco leaves with green or black colors, higher proportion of superior tobacco, and increased average selling price.

Table 3. *Analysis of the appearance quality of tobacco leaves.*

	Orange tobacco proportion (%)	Lemon tobacco proportion (%)	Green or black tobacco leaves (%)	Proportion of superior tobacco (%)	Average price of cured tobacco (Yuan/kg)
Barn A	81.8	14.6	3.6	41.6	22.4
Barn B	81.4	14.8	3.8	41.5	22.3
Barn C	76.5	19.2	4.3	37	20.5

Note: Yuan - Monetary unit in China.

Discussion

New device technology and precision control tobacco curing

The new device, which is a mature electrical industrial product integrated with solar collector, heat pump heating and heat pump dehumidification technology, used in this study can accurately and steadily control the dry-bulb and wet-bulb temperature of air according to curing process parameters, which is particularly advantageous for the synthesis and degradation of inner substances in tobacco leaves. Empirical addition of fuels and fuels combustion characteristics can adversely affect tobacco curing in production (Frey et al., 2003). Typically, heat for tobacco curing is provided by burning of fuels, such as coal or firewood, through manual labor and automatic fuel combustion under natural conditions (Truong et al., 2016; Tippayawong et al., 2006), which bring the large difference between the actual dry and wet-bulb temperature and target dry and wet-bulb temperature of the barn during tobacco curing process. This result is also consistent with the observation of Gong and Pan (2003) using a precise control equipment to cure tobacco.

Utilization prospect of solar energy resources in China

Solar irradiation and collecting area are the two main factors effecting solar-energy collection efficiency (Chow, 2010). *Figure 6* shows the distribution of solar radiation and the tobacco growing regions in China. From July–September, the daily total radiation at the experimental station in Xuchang was 18.55–23.33 MJ/m^2, which belongs to a general resources region. In certain provinces, such as Yunnan and Liaoning, solar energy resources are more abundant, and the design and installation of solar energy collectors at the top of barns should be considered for tobacco curing. Solar collection efficiencies above 94 % have recently been achieved (Sabiha et al., 2015; Verma et al., 2015) and many policies that encourage use of green energy have been issued by the government (Wang et al., 2015; Xiao et al., 2015). These have great significance for fossil-fuel energy savings in the tobacco curing industry in the near future.

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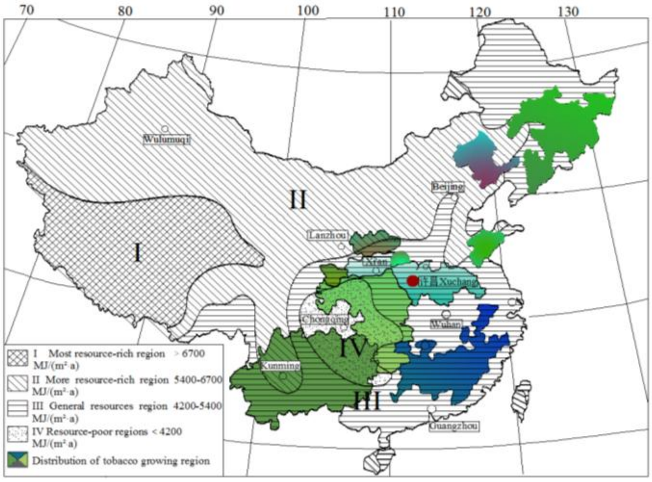

Figure 6. *Distribution of solar radiation and tobacco growing regions in China.*

Note："●" Location of the experimental point. The data sources are obtained from China's solar energy distribution and China tobacco plan.

Conclusion

This study proposed an integrated energy-heating equipment that could accomplish preferential solar energy assisted tobacco heating, which primarily consisted of heat pump heating and utilization of heat recycled from heat pump dehumidification. The equipment has the advantages of compact combination, precise control, and it is suitable for the popularization of a few tobacco planting areas. The experimental results show that in general resource regions with sunny days or partly cloudy days, solar energy could be efficiently used to provide heat for tobacco curing during the entire curing process. Heat pump dehumidification technology can discharge excess water in barns and recycle the heat from high temperature moist air. Using the equipment to cure tobacco can improve the appearance quality of the tobacco leaves and reduce the curing cost per kg of dry tobacco by 21% when contrasted to coal-fired curing. Thus, this device that can remove about 3500 kg of water in 142 h from this study may be suitable to use for drying other agricultural products after transformed.

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PROTEIN BAITS, VOLATILE COMPOUNDS AND IRRADIATION INFLUENCE THE EXPRESSION PROFILES OF ODORANT-BINDING PROTEIN GENES IN *BACTROCERA DORSALIS* (DIPTERA: TEPHRITIDAE)

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Abstract. Chemical communication in insects is based on odorant-binding proteins (OBPs), but the molecular mechanisms by which these OBPs allow the perception of volatiles and host chemicals remain unclear. *Bactrocera dorsalis* (Hendel) is one of the most economically important pests of fruits and vegetables. In this study, the OBPs of *B. dorsalis* (BdorOBPs) were evaluated in response to different attractive protein baits, brewer's yeast volatile compounds and irradiation. The expression of 10 genes encoding OBPs in the antennae of *B. dorsalis* during three adult physiological life stages (pre-mating, post-mating and post-oviposition) were analysed. All selected BdorOBP genes were found to contain one conserved pheromone-binding protein/general-odorant-binding protein domain (PBP-GOBP), except OBP8, which contained two of these conserved domains. An unrooted phylogenetic tree was constructed to show the relationships among these 10 BdorOBPs and the OBPs belonging to the same OBP family in other insects. We found significantly different transcript expression profiles in each OBP gene at each stage in response to different treatments; these results revealed that OBP2 expression was significantly increased in response to baits at each adult physiological stage, while OBP2, OBP5 and OBP1 were highly expressed in response to combined-volatiles treatment at all tested physiological stages. OBP3, OBP5 and OBP10 showed high expression in response to irradiation at all tested physiological stages. Thus, we infer that protein baits, brewer's yeast volatiles and irradiation significantly influence the transcript levels of OBP genes, which may act in olfactory perception.
Keywords: *transcript analysis, irradiation, brewer's yeast volatiles, oriental fruit fly, insect olfaction*

Introduction

Bactrocera dorsalis is the most devastating pest in several Asian countries including China. This fruit fly was reported for the first time in Taiwan in 1912 (Hardy, 1973). *B. dorsalis* is a serious quarantine pest throughout the world, resulting in severe financial damage to multiple crops that are among its 270 host plant species (Clarke et al., 2005; Chen et al., 2008; Leblanc et al., 2013; Vargas et al., 2015). The extensive damage caused by this particular pest worldwide raises serious concerns about crop yields due to the high resistance of this pest to chemical insecticides (Hu et al., 2014). A variety of methods such as the sterile insect technique, mass trapping, protein bait spraying and male annihilation have been developed to suppress or eradicate *B. dorsalis* and related species throughout Asia and the Pacific regions (Jessup et al., 2007; Mau et al., 2007; Piñero et al., 2009a; Piñero et al., 2009b; Vargas et al., 2015).

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Chemoreception is an important characteristic in the selection of host plants by phytophagous insects. Odorant-binding proteins (OBPs) present in the olfactory system play an essential role in sensitivity to odorants, such as pheromones and host chemicals (Zheng et al., 2013). The olfactory system in insects consists of three central types of proteins: OBPs, odorant receptors (ORs) and odorant-degrading esterases (ODEs) (Justice et al., 2003). Among these proteins, OBPs frequently function in the first step of odour perception. The process of chemical communication in both insects and vertebrates is performed by OBPs (Tegoni et al., 2004; Dahanukar et al., 2005; de Bruyne and Baker, 2008). Insects possess a notably complex and cosmopolitan olfactory system, through which they are potentially able to detect a variety of external volatile compounds including plant volatiles and gender-specific mating pheromones (Hsu et al., 2012; Zheng et al., 2012b; Papadopoulos et al., 2013; Zheng et al., 2013; Liu et al., 2015). Insect OBPs may represent an important molecular target in the application of eco-friendly pest management approaches, as they are proposed to be involved in olfactory cue discrimination due to their intense expression in antennal sensillum lymph (Qiao et al., 2009; He et al., 2011; Sun et al., 2013a, 2014; Pelosi et al., 2014). Insects usually use their antennae as olfactory organs in order to detect the odours of plants, fruits and chemicals in the outside environment (Sun et al., 2016a).

Conversely, many studies have shown that the expression of OBPs is not limited to the olfactory organs; thus, their physiological functions may be broader and more difficult to understand than expected (Park et al., 2000; Foret and Maleszka, 2006; Li et al., 2015; Sun et al., 2012; Yuan et al., 2015). To determine whether the OBPs in the antennae of *Bactrocera* species respond to volatiles, the present study focuses on the expression patterns of 10 OBPs in the antennae of *B. dorsalis* (BdorOBPs) in response to protein baits, volatiles and irradiation. Insect OBPs, which are specifically or primarily expressed in the antennae, have been proposed to play roles in olfactory function in response to different kinds of attractive host plant volatiles (Zhou, 2010; Leal, 2013)**.** Previously, an electroantennogram (EAG) was employed to record the electrophysiological responses of the antennae of *B. dorsalis* to spent brewer's yeast volatiles, alcohols, aldehyde alcohols and aldehydes to determine the olfactory response of the antennae towards chemical odours. The results of that study revealed that volatiles could potentially evoke higher relative EAG values in the antennae of *B. dorsalis* during three adult physiological stages (Jang and Light, 1991; Du et al., 2015). A total of 20 BdorOBP genes have been identified from the *B. dorsalis* transcriptome; however, only 10 BdorOBP*s* have been characterized (Zheng et al., 2012b, 2013).

B. dorsalis transcriptomes and expression analysis in different tissues have been analysed, but the study of the molecular basis of olfaction in response to different stimuli is still in its initial stages (Shen et al., 2011a; Geib et al., 2014). In the studies mentioned above, scientists have reported the EAG response of *B. dorsalis* to different chemical odours and identified OBP genes that are responsible for the first step in the perception of chemical odours. However, few studies have evaluated the expression profiles of the different OBP genes of an insect pest in response to different volatiles, particularly different baits, spent brewer's yeast volatiles and irradiation. Hence, there is a pressing need to study the influence of different kind of volatiles on the expression levels of OBP genes in the antennae of *B. dorsalis.*

Therefore, the current study was based on the hypothesis that different protein baits, spent brewer's yeast volatiles and irradiation would influence the expression levels of OBP genes at three adult physiological stages in the antennae of *B. dorsalis.* The objective of the study was

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to evaluate the effects of different protein baits, spent brewer's yeast volatiles and the application of radiation on the transcript levels of the 10 previously identified BdorOBP genes in the antennae of *B. dorsalis* during the pre-mating, post-mating and post-oviposition stages. The findings of the present study may provide knowledge of the molecular mechanisms of action of the variety of OBPs present in the antennae of *B. dorsalis* and potentially suggest novel tactics for the suppression of *B. dorsalis*. A greater understanding of the mechanisms underlying OBP function may allow the invention of novel control methods to disrupt these internal physical mechanisms, perhaps by influencing the gene expression profiles of OBP genes and thus support novel strategies for long-term effective management of tephritid fruit fly species.

Material and Methods

Insect rearing

The insects were reared at the Institute of Beneficial Insects, Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, China, under controlled conditions (26±1°C; 60-70% RH; 12 hr light/12 hr dark photoperiod) for approximately 60 generations. The eggs of *B. dorsalis* were collected using a homemade ovipositional bottle including artificial juice and then were placed on a single layer of wet tissue paper in a larval diet tray. The adult flies were reared on artificial diet prepared with yeast extract and sugar in accordance with the procedures described by (Spencer and Fujita, 1998).

Experimental materials

Proteins were prepared at the Institute of Beneficial Insects at Fujian Agriculture and Forestry University, Fuzhou, Fujian Province, China, while torula yeast was obtained from Baifude Biotechnology, Wuxi, Jiangsu. Spent brewer's yeast volatile compounds phenyl acetate, ethyl caprylate, and octyl acetate (analytical reagents) were purchased from Solarbio Pvt. Ltd., Shanghai, while paraffin oil (100%) was obtained from Acros Organics (Geel, Belgium).

Experimental methods

Protein baits and volatile regulation for female and irradiation application method for male B. dorsalis

This experiment was planned to evaluate the effects of protein baits and torula yeast on the transcript expression of 10 previously identified full-length cDNAs of OBP genes in the antennae of *B. dorsalis* at different adult life stages: pre-mating (2-3 days), post-mating (7-9 days) and post-oviposition (11-13 days). A total of one hundred (100) mature females were released in a 33 cm × 33 cm × 33 cm cage and starved for 12 hrs overnight prior to feeding using protein bait and torula yeast. The insects were treated with two different protein baits (1:10 dilution with paraffin oil), whereas the control group was treated with an equal volume of normal protein diet (Zheng et al., 2012b). Three spent brewer's yeast volatile compounds and their blend mixtures were prepared: 15% paraffin oil (Control, Ck), 15% (75 µl phenyl acetate + 425 µl paraffin oil), 25% (125 µl ethyl caprylate + 375 µl paraffin oil), 15% (75 µl octyl acetate + 425 µl paraffin oil) and a mixture of all compounds (15% phenyl acetate + 25% ethyl caprylate + 15% octyl acetate) (MIX). Treatments were denoted by Ck: control, PA: phenyl acetate, EC: ethyl caprylate, OA: octyl acetate, MIX: mixture of three volatile compounds (PA + EC

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+ OA). *B. dorsalis* was irradiated with ^{137}Cs at 100 Gy for 1, 2 and 3 days at the pupal stage, and kept at 25 ± 1 °C, RH 65% ± 5%, and L:D = 12:12 after adult emergence for 1 day, 6 days and 8 days. Afterwards, 100 infertile males were collected on each day, and their antennae were dissected.

RNA isolation and qRT-PCR analysis

Adult tissue samples were dissected from 500 individuals. The total RNA for each group was extracted from the antennae of *B. dorsalis* at 2-3 days (pre-mating stage), 7-9 days (post-mating stage) and 11-13 days (post-oviposition stage) by using a Tiangen RNeasy Kit. Two micrograms of total RNA was reverse-transcribed by the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Japan) using the oligo dT primer. qRT-PCR was conducted to detect the expression of genes encoding OBPs by using gene-specific primers (*Table 1*), Top Green qPCR Super Mix (Transgen, China) and a real-time thermocycler (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. For internal standardization, β-*Actin* was amplified. Prior to starting the gene expression analysis, the PCR efficiency of the genes was validated. The qRT-PCR was performed in a volume of 20 µl containing 10 µl of 2× TransStart® Top Green qPCR Super Mix, 2 µl cDNA (1:100 diluted), 0.8 µl of each (forward and reverse) primer and 6.4 µl nuclease-free H_2O. The qRT-PCR programme was: 94°C for 30 s followed by 40 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 10 s. The melting curve was analysed for the PCR products to confirm the presence of a single amplified fragment at the end of each PCR. The β-actin gene was used as a housekeeping gene to standardize the qRT-PCR results. A relative quantitative computing method ($2^{-\Delta\Delta Ct}$ method) was used to compute gene expression (Livak and Schmittgen, 2001). Three biological replicates were performed.

Table 1. *Primers used for gene expression detection of BdorOBPs by qRT-PCR*

Primer name	(5'→3') Nucleotide sequence
BdorOBP1RTF	GGCGAGCGTTGTTTCCAG
BdorOBP1RTR	ATCGGCACCAGCACTTCC
BdorOBP2RTF	TTCTTCGCTGTTGCTGTTTT
BdorOBP2RTR	AGAAGCATTTGACTTTGCCATC
BdorOBP3RTF	TTGACCGAGGAGCAGAAAC
BdorOBP3RTR	TTGACTTTGCCATCGACTT
BdorOBP4RTF	GAACTTCTTCGCTGTTGCTG
BdorOBP4RTR	CTTGACTGTCATCGCCTTTG
BdorOBP5RTF	GGATTTCCACGACTTCATTGAG
BdorOBP5RTR	CTTTAGCCGCATCAGGTTTG
BdorOBP6RTF	ACGAAGCCAAAGTCACGG
BdorOBP6RTR	GCATCAAAGACGCCATCC
BdorOBP7RTF	GTTGCAGGCAAATTCCAGAT
BdorOBP7RTR	AGAGTCCCATTCGCTCCACA
BdorOBP8RTF	ACGGCTAAGGCTCTAAAGAACC
BdorOBP8RTR	AAACCACTTGTGACGCTTCG
BdorOBP9RTF	GCGATGCCGACCATGATGAC
BdorOBP9RTR	ACCACCACGATAAGCCCACT
BdorOBP10RTF	GTGTCTTCTGCGAACATGAGG
BdorOBP10RTR	CACTTGTGACCACGATAGGC
β-actinRTF	CTCGTCCAACCGTTCATACC
β-actinRTR	CTGACCTGCCCACTGAAGTT

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Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software package (IBM Corporation, Somers, NY, USA) to calculate *Dunnett's test* for comparing control with other treatments (SPSS for Windows, Version 16.0, 2007).

Results

Identification of PBP-GOBP domains in BdorOBP genes

All 10 BdorOBP genes were found to contain one conserved PBP-GOBP domain, except for OBP8, which, remarkably, contained two such conserved domains. In the OBP family, the amino acid sequence residues of each BdorOBP gene were green when compared with the hidden Markov model (HMM), which indicates a very high-quality hit. BdorOBP conserved domains are presented in (*Figure 1*), while the BdorOBP genes along with their accession numbers are presented in (*Table 2*). The PBP-GOBP conserved domain was identified by using the Pfam database (version 31.0) (Finn et al., 2016).

<BdorOBP1|PCI domain|

NADYEEKTEDDFLSAGERCFQRERLAASYQRRFDNFDYPDEEPV
QRYVHCIWTELKLWNDRTGFNVEHIAALYRDKANTEVLVPILSDC
NRNAQNEPTLKWCYRAFKCVLNS

<BdorOBP2|PCI domain

YPPPELLKELQPVHDSCVAKTGVTEEAIKEFSDGDVHEDELLKCYM
YCVFEETDVLHEDGEVHLEKILDKLPESMHVIALHMGKKCLYPKGD
NKCERAFWLHR

<BdorOBP3|PCI domain|

GKLTEEQKQKVHAAAAECFKETGASEDAVRALLKGDDSQVDGKVK
CFAKCTLGKLDLLQNGKVNEEKVQNILGKLIGEEKAKAAQAKCNGL
KGTDECDTAYQIRQCYAAG

<BdorOBP4|PCI domain|
EGMLTPEQIQKVHTLSNECLKETGASEDAIRALIKGDDSQVDGK
VKCYAQCMLVKLGYVENGKVNEEKVQNILGKLIGEEKAKATQ
AKCNGLKGTDECDTAYQIRQCYSAG

<BdorOBP5|PCI domain|

PHDPEMRGYIEDCNKEHNVSPKDFHDFIEGKLTTVPENMKCSSQC
IMVKQGIMDESGNFKPDAAKAKMKEDKLVAAVDECKDLSGSTPC
DTAFKITSCMLSK

<BdorOBP6|PCI domain|

AVCQLPADLEKFHKACMDEAKVTDEQMRQFFQNGMKASDATENI
KCQMKCMMQKQGIWKDGVFDADAKIKELVQNPKFKGKEARTNK

<BdorOBP7|PCI domain|

GKFQIRTAQDALDAHEACHEEYRVPEDIYQKFLNYEFPAHKRTNCY
VKCFVERMGLFTEEKGFDEKAIIAQFTAKSSKNLAKVSHGLEKCLDH
NEHDSDTCTWANRVFSCWIS

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<BdorOBP8|PCI domain|
ATTKAAEQDVDSDTEILRKCLREVGSKDLVGELQKVARYSKW
TSEEVPCFTRCLASMKHWFDADESKWNKQQIADDLGADMYN
YCRYELDRYNEDSCEFAYTGLRCLKQA

TLETYKNIVSCASELNVTMKELQKYAAFPTKEVVPCLFQCLAE
KMNFYTPTYEWNLDNWVQAFGPMRQDRTASNVCKVSAEHM
KTRDKCEWMYEEYNCLER

<BdorOBP9|PCI domain|
SAEYVVKNEENLQQYRRECATELKVPAEHIEQFRKWQFPNDA
VTQCYLKCVFEKFGLFDAVTGFNVEHIHQQLQGAEVAPPGDA
DHDDVVHDKIAACVDTNEQGSNACEWAYRGGVCFIKE

<BdorOBP10|PCI domain|
DEEWVPKNVAQIKAIRQECIKDFPLSEEYIQKMKNFEYPDEEPVR
KYLLCTAKKLGVFCEHEGYHADRVAKQFKMDLDEAEVIAIAEG
CADKNVEGSSADVWAYRGHKCVMAS

Figure 1. *BdorOBPs contain one conserved PBP-GOBP domain, which is a typical characteristic of OBPs. All BdorOBP genes contain one conserved PBP-GOBP domain, except OBP 8, which contains 2 of these conserved domains. The PBP-GOBP domains were identified by using the Pfam database (version 31.0), and the amino acid sequence residues of each BdorOBP gene were green when compared with the hidden Markov model (HMM), which indicates a very high-quality hit.*

Table 2. *BdorOBP genes with their accession numbers and nucleotide lengths*

Gene name	Accession number	Length
BdorOBP*1*	*KC559112*	*159*
BdorOBP2	*KC559113*	*138*
BdorOBP*3*	*KC559114*	*146*
BdorOBP*4*	*KC559115*	*136*
BdorOBP*5*	*KC559116*	*130*
BdorOBP*6*	*KC559117*	*104*
BdorOBP*7*	*KC559118*	*142*
BdorOBP*8*	*KC559119*	*274*
BdorOBP*9*	*KC559120*	*148*
BdorOBP*10*	*KC559121*	*149*

Phylogenetic analysis of insect OBPs

A phylogenetic tree was generated by obtaining the amino acid sequences of the ten putative OBPs of *B. dorsalis* from the work of (Zheng et al., 2013). The phylogenetic tree was built to demonstrate the relationships of the 10 BdorOBPs to 140 OBP sequences from 7 different orders and families of insects that possess the same OBP family, including 20 OBP sequences from *Apis mellifera* (Hymenoptera: Apidae), 7 OBP sequences from *Bombyx mori* (Lepidoptera: Bombycidae), 30 OBP sequences

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from *Drosophila pseudoobscura* (Diptera: Drosophilidae), 29 OBP sequences from *Halyomorpha halys* (Hemiptera: Pentatomidae), 15 OBP sequences from *Spodoptera litura* (Lepidoptera: Noctuidae), 10 OBP sequences from *Tenebrio molitor* (Coleoptera: Tenebrionidae) and 44 OBP sequences from *Tribolium castaneum* (Coleoptera: Tenebrionidae), as shown in (*Figure 2*). This phylogenetic tree clarified the connections between *B. dorsalis* and other insects in the groups listed above. Amino acid sequences were used to build an unrooted phylogenetic tree using MEGA 7.0 with the pairwise deletion option under the JTT empirical amino acid substitution model (Kumar et al., 2016). Branch support was assessed by bootstrap analysis with 1000 replicates.

Figure 2. *Unrooted phylogenetic tree of candidate OBPs from Bdor (B. dorsalis) and seven additional insect species with available amino acid sequences (Amel: Apis mellifera, Bmor: Bombyx mori, Dpse: Drosophila pseudoobscura, Hhal: Halyomorpha halys, Slitura: Spodoptera litura, Tmor: Tenebrio molitor and Tcas: Tribolium castaneum). The highly divergent signal peptides in the N-terminal region were removed, and a neighbour-joining tree was constructed by using MEGA 7 with the pairwise deletion option under the JTT empirical amino acid substitution model. Only bootstrap values above 50% are shown. The black diamonds indicate the 10 B. dorsalis OBP genes.*

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Effects of bait and torula yeast on BdorOBP transcript levels in females

The qRT-PCR results revealed that all BdorOBPs showed significant changes in expression in response to protein bait and torula yeast except OBP4, OBP8 and OBP9, as shown in (*Figure 3a*). However, BdorOBP3 showed no significant expression in response to starvation (*Figure 3a*).

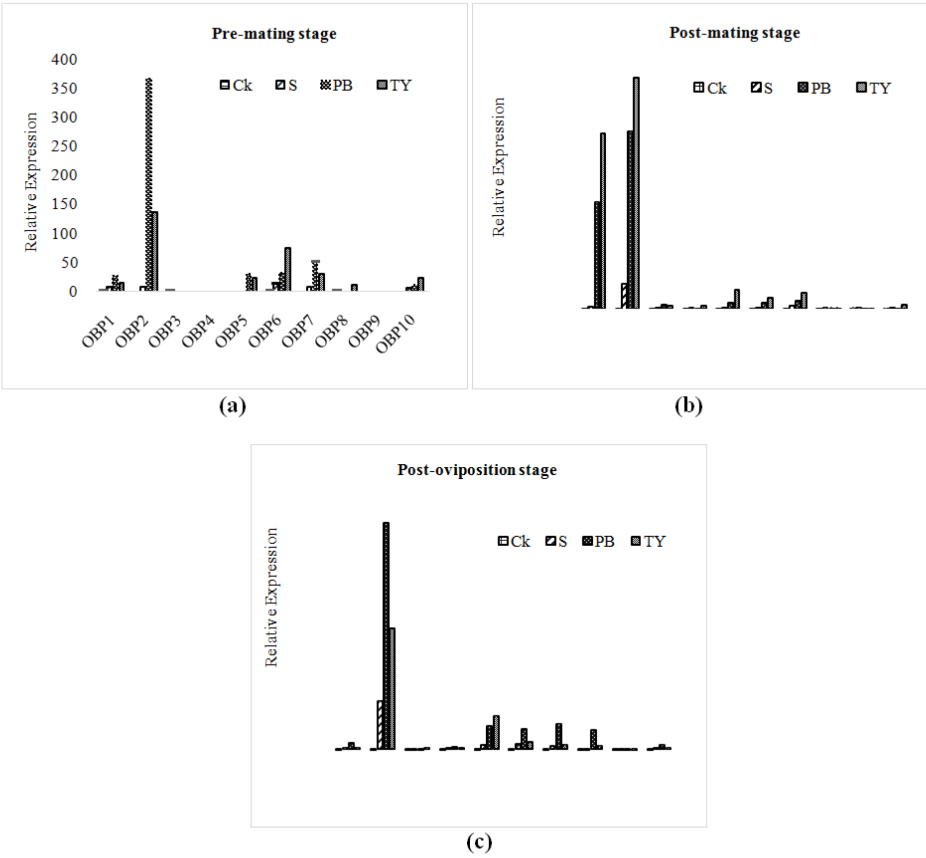

Figure 3. *Transcript expression analysis by qRT-PCR of the ten BdorOBP genes in the antennal tissues of mature adult females in response to protein bait and torula yeast. (a) Pre-mating stage; (b) post-mating stage; (c) post-oviposition stage. Treatments were denoted by Ck: control, S: starvation, PB: protein bait, TY: torula yeast. Analysis of variance (ANOVA) indicated a significant difference in the expression levels of OBPs at p < 0.05. However, OBP3 was expressed weakly under starvation treatment, and hence, ANOVA indicated that this difference was not significant (Figure 3a). The ANOVA indicated a significant difference among the expression levels of all BdorOBPs (p < 0.05, Figure 3b), except those of OBP9 and OBP10. However, OBP6 in response to starvation and OBP8 to starvation and protein bait were not highly expressed (Figure 4b). The ANOVA indicated a significant difference (p < 0.05) among the expression levels of all BdorOBPs (Figure 3b), while OBP1 in response to starvation and torula yeast; OBP4, OBP8, OBP9 to starvation; and OBP10 to starvation and torula yeast were not expressed significantly (Figure 3c).*

The expression levels of OBP1, OBP2, OBP5, OBP6 and OBP7 were higher in insects given protein bait and torula yeast than those of OBP3 (*Figure 3b*). OBP6 and OBP8 exhibited no significant expression in response to starvation and protein bait treatments, respectively ($p > 0.05$, *Dunnett's test*) (*Figure 3b*). However, the

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transcript levels of OBP4, OBP9 and OBP10 were not altered significantly (p > 0.05) in response to any of the treatments (*Figure 3b*). Protein bait and torula yeast increased the transcript level of OBP6, while all of the remaining OBPs showed no response to protein bait and torula yeast, as shown in (*Figure 3b*). The expression levels of OBP1 and OBP10 were significantly increased in the antennae of *B. dorsalis* in response to starvation and torula yeast, while those of OBP8 and OBP9 showed no response to starvation. OBP4 showed no change in expression in response to the baits at any of the tested stages (*Figure 3c*). These results suggest that different baits may significantly alter the transcript profiles of some BdorOBPs, which may play an important role in altering the mechanism of insect olfaction.

Effects of volatiles on BdorOBP transcript levels in females

The analysis of the volatile treatments indicated relatively low expression levels in OBP5, OBP8, OBP9 and OBP10, while the remaining BdorOBPs showed significantly higher expression in all treatments, as shown in (*Figure 4a*).

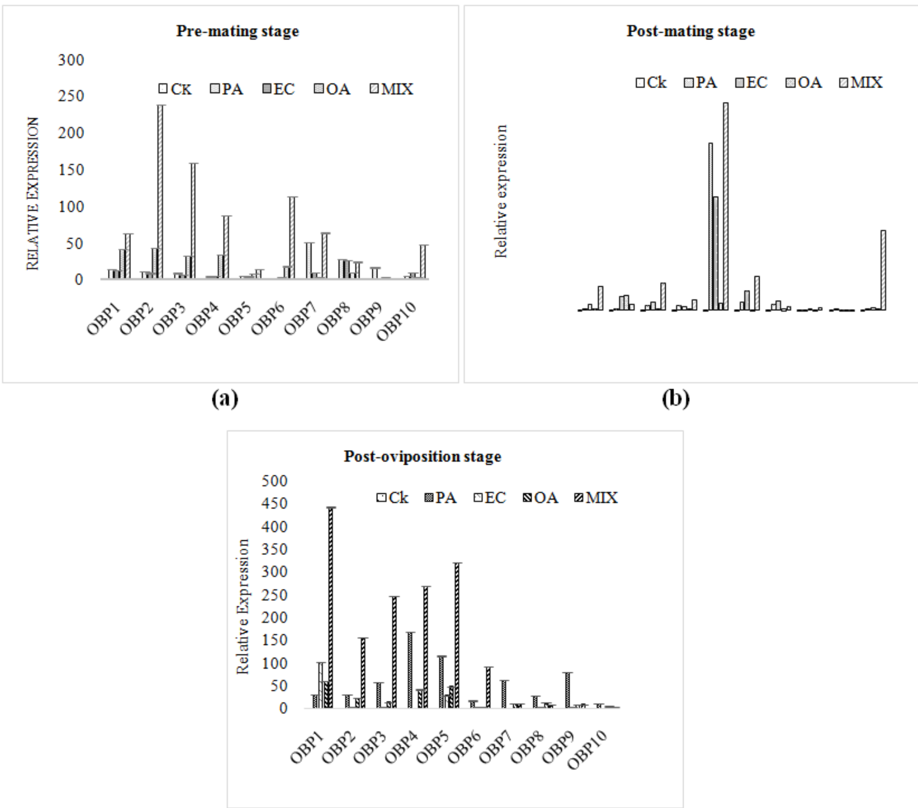

Figure 4. *Transcript analysis of the ten BdorOBP genes in the antennal tissues of mature adult females in the (a) pre-mating stage; (b) post-mating stage; and (c) post-oviposition stage. Treatments were denoted by Ck: control, PA: phenyl acetate, EC: ethyl caprylate, OA: octyl acetate, MIX: mixture of three volatile components (PA + EC + OA). Relative transcript levels were calculated using β-actin as the standard. A significant difference in the expression levels of BdorOBP genes was found by Dunnett's test ($p < 0.05$). OBP6 in response to starvation (Figure 4a), OBP8 to PA and EC (Figure 4b), OBP6 to OA, and OBP10 to PA and EC were not expressed significantly (Figure 4c).*

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Interestingly, higher expression levels were observed in response to MIX and OA at all tested stages, as shown in (*Figure 4a, 4b* and *4c*). Only OBP*5* exhibited a significant expression change in response to all treatments, as shown in (*Figure 4b*). In contrast to the lower expression levels observed in the last five BdorOBPs (OBP6-OBP10), the first five BdorOBPs (OBP1-OBP5) showed significantly increased expression in response to all treatments, particularly MIX and PA, as shown in (*Figure 4b*). OBP6 showed no change in expression level in response to PA and OA, as shown in (*Figure 4a, 4b*), while OBP8 and OBP10 also showed no change in response to PA and EC, as shown in (*Figure 4b, 4c*).

Effects of irradiation on BdorOBP transcript levels in males

The qRT-PCR results showed that the transcript levels of the BdorOBPs changed significantly at all tested stages with a ^{137}Cs irradiation dose of 100 Gy. The expression levels of the BdorOBPs were altered significantly in response to irradiation, except those of OBP3, OBP4 and OBP7. The expression levels of OBP2, OBP5, OBP7 and OBP9 were significantly increased, as shown in (*Figure 5a*).

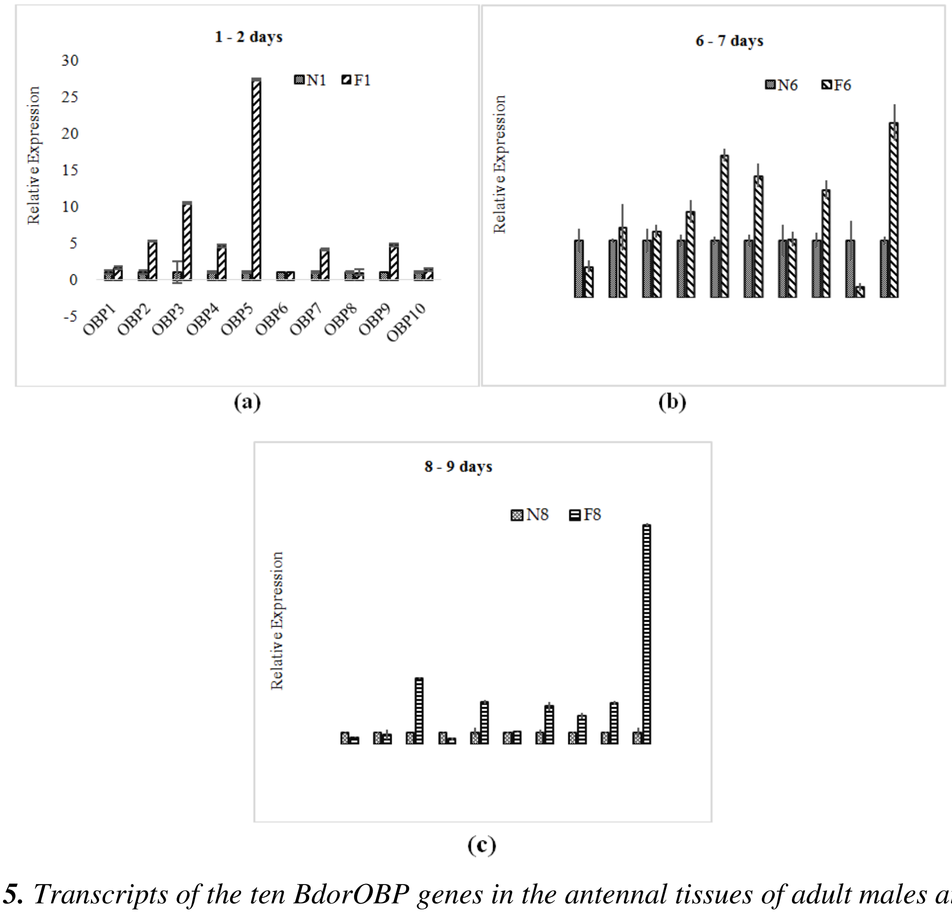

Figure 5. *Transcripts of the ten BdorOBP genes in the antennal tissues of adult males at (a) 1-2 days; (b) 6-7 days; (c) 8-9 days. The treatments were denoted by N: normal male and F: irradiated (infertile) male. Relative transcript levels were calculated using β-actin as the standard. A significant difference among the expression levels of all BdorOBP genes was found at p <0.05. Low expression levels were observed in OBP3, OBP4, and OBP7 (Figure 5a), while OBP1 did not show significant expression (p > 0.05, Figure 5b, 5c).*

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However, each OBP was significantly differentially expressed, except OBP1, which showed no change. OBP4, OBP5, OBP6, OBP8, and OBP10 exhibited particularly high expression in response to irradiation, as shown in (*Figure 5b*). OBP3, OBP5, OBP7, OBP8, OBP9 and OBP10 showed exceptionally abundant expression in contrast to the other OBPs, as shown in (*Figure 5c*). However, OBP1 showed no response to irradiation, as shown in (*Figure 5b*, *5c*).

Discussion

Insects essentially depend on the antennae to distinguish plant volatiles or sex pheromones in the environment (Steinbrecht, 1997); hence, we selected *B. dorsalis* antennae to evaluate the expression profiles of 10 BdorOBP genes in response to protein baits, brewer's yeast volatiles and irradiation. Previously published papers have reported functional studies of insect OBPs in chemoreception since their discovery in 1981, but the exact functions of insect OBPs in response to different stimuli present in the environment are still unknown (Zhou, 2010; Leal, 2013). Highly developed olfactory systems are present in phytophagous insects, allowing them to use numerous chemical stimuli to identify plant hosts (Pelosi et al., 2006). The OBPs in the olfactory signal reception pathway play an essential role in the transportation of specific odorants to ORs to stimulate insect behaviours (Pelosi et al., 2006). Hence, OBPs are thought to be directly essential for odorant perception and represent potential targets for pest control via insect attraction.

B. dorsalis antennae have a strong EAG response to spent brewer's yeast volatiles (Du et al., 2015), and the results of the present study revealed that OBP2, OBP5 and OBP1, which are present in the antennae of *B. dorsalis*, were highly expressed in response to spent brewer's yeast volatiles at all tested stages. OBPs show significant attraction responses to different volatiles (Cornelius et al., 2000; Hu et al., 2007; Liu et al., 2010; Shen et al., 2011b). Another author has reported that some volatile compounds, such as benzaldehyde, showed very weak attraction effects in all adult physiological stages of female *B. dorsalis* (Ling, 2013), and the results of our study also showed that some OBPs were down-regulated, perhaps due to the weak affinity of volatiles to some OBPs. A similar phenomenon was observed in the case of ester alcohols, the major constituents of aromatic odour in most fruits, which plays an essential role in the attraction behaviour of *B. dorsalis* (Zhang et al., 2007). Tephritidae have shown significant differences in their responses to other esters, such as beer waste yeast, hydrolysates, and volatile compounds of the other 3 kinds of esters (Du et al., 2012).

Previously, thirty-four compounds were selected to investigate the interaction of OBPs with rice plant volatiles (Lou et al., 2005; Sun et al., 2013b). *Cnaphalocrocis medinalis* OBP2 (CmedOBP2) showed a significant response to host volatiles, whereas CmedOBP3 showed relatively little response to these volatiles (Sun et al., 2016b); our study found that all BdorOBP genes showed low or no response to combined volatile treatments at all tested physiological stages, except the OBP2, OBP5 and OBP1 genes, which were highly expressed. Because all insects have different OBP sequences and each OBP family might perform a different function, different OBPs can display different mechanisms in response to different kinds of volatiles present in plants and fruits (Sun et al., 2016b).

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A previous report indicated that synthetic volatile compounds targeted OBP3 or OBP7, which were confirmed to be accountable for (*E*)-ß-farnesene sensitivity, and provoked significant behavioural responses in aphids (Sun et al., 2012), while the results of our studies revealed that the first five BdorOBP genes (OBP1-OBP5) were significantly expressed in response to all treatments, specifically to MIX and PA, and weak expression was observed in the last five BdorOBP genes (OBP6-OBP10) in response to volatiles.

The prerequisite for the illumination of the molecular mechanism of olfaction is the documentation of the genes accountable for volatile discrimination. Candidate olfaction genes have been recognized in numerous other species through annotation and antennal transcriptome analysis. Although *B. dorsalis* olfaction research has previously lacked transcriptome data for the classical olfactory organs, specifically the antennae, several studies of the *B. dorsalis* transcriptome have recently been conducted (Shen et al., 2011b; Zheng et al., 2012a; Geib et al., 2014). However, limited studies have investigated the effects of the volatile compounds reported for control of *B. dorsalis* on OBP gene expression profiles in *B. dorsalis* and other insect pests. In light of the present findings, the fact that *B. dorsalis* males and females were captured in greater numbers in torula yeast bait traps than in protein bait traps likely reflects a lower availability of proteinaceous food in the habitats sampled by (Siderhurst and Jang, 2010; Royer et al., 2014).

Insect OBPs are responsible for the detection of host plant attractants. A single OBP may participate in the recognition of several chemical odours (Vogt et al., 1991; Pelosi et al., 2014); however, our results reported that only OBP2 was significantly expressed in response to baits at each adult physiological stage among these 10 OBP genes. On the other hand, specific compounds might be recognized by more than one OBP gene, as the results of our study revealed that few OBP genes showed significant responses to spent brewer’s yeast volatiles, while others showed no response. To the best of our knowledge, our data exhibit the relationship of these ten OBP genes to spent brewer’s yeast volatiles, protein bait and irradiation for the first time.

The combination of different OBPs in insects may form a molecular basis for the ability to identify and distinguish relevant olfactory cues amid the vast spectrum of odorants emitted from fruits or plants (Takken and Knols, 1999; Zwiebel and Takken, 2004). In our study, some OBPs showed the same response to MIX treatment and were highly expressed at all physiological stages. This result further supports the view that OBPs are not just simple, general solubilizers that act as transporters for odorants but also play a vital role in the perception of different kinds of chemicals in the environment (Pelosi et al., 2006; Swarup et al., 2011). An OBP in *Hylamorpha elegans* showed a high binding affinity to β-ionone (Venthur et al., 2016), a volatile compound derived from plant carotenoids (Simkin et al., 2004).

Most of the OBP genes showed significant responses to MIX treatment at all adult life stages (*Figure 4a, 4b* and *4c*). Irradiation has been reported to significantly destabilize fly fitness by damaging tissues and interior organs to some degree, inducing metabolic disorders (Lauzon and Potter, 2012; Yao et al., 2017), and our results showed that irradiation significantly altered the transcript levels of OBP genes at all adult life stages (*Figure 5a, 5b* and *5c*), with especially abundant expression in the second and third stages (*Figure 5b, 5c*). These significant observations suggest that the effects of applied treatments on the transcript levels of BdorOBP genes may play an essential role in understanding the insect olfaction mechanism in response to different kinds of

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protein baits, volatiles released by different kinds of plants in the environment and irradiation applied to insects at the pupal stage.

Our results provide a direct BdorOBP transcript analysis that represents new evidence for the role of olfactory proteins in the reception of protein baits and volatiles. We believe that these findings establish a foundation for understanding the molecular mechanisms by which OBPs contribute to olfactory recognition and have applications for integrated pest management techniques. However, an urgent need exists to identi characterize the remaining ten OBP genes of *B. dorsalis* in order to understand the transcriptional regulation of all OBPs in response to protein baits, volatiles and irradiation. The functional study of antennae-specific or antennae-enriched OBPs can assist us to better understand the involvement of OBPs in the mechanisms of insect olfaction with respect to different environmental stimuli and to design an odorant-based insect control strategy for the management of *B. dorsalis*.

Conclusions

Since its arrival in mainland China, *B. dorsalis* has become one of most destructive pests in orchards. Furthermore, this pest is presently spreading to more suitable areas of North China. The use of chemical insecticides remains the foremost way to control *B. dorsalis* in most developed countries, especially in China. In previous decades, various approaches have been established to monitor its detection and dispersal in order to minimize damage, and many studies have been carried out to interpret different aspects of its invasion mechanism. Further studies are still needed to understand its adaptation to new environments, resistance to chemicals, and high procreative ability. For the first time, we have evaluated the effects of different kinds of attractive baits, brewer's yeast volatiles and irradiation on the transcript expression levels of 10 OBP genes in the antennae of *B. dorsalis* during three adult physiological stages. Some BdorOBP genes are significantly up-regulated in response to the applied treatments, and others may play a vital role in mediating the response of males to irradiation, while OBP genes in females showed high transcript levels in response to different baits and brewer's yeast volatiles.

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Conflicts of Interest: The authors declare no conflict of interest.

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THE EFFECT OF DIFFERENT AMERICAN VINE ROOTSTOCKS ON THE PHYTOCHEMICAL CHARACTERISTICS IN DRIED BANAZI BLACK GRAPE (*VITIS VINIFERA* L.)

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Abstract. Banazi Black grape which is almost never consumed freshly is a very important, seed bearing, local type which is directly dried in the sun with its cluster without any processing. Ungrafted (5 years old and over 40 years old) and grafted (41B, 99R, 1103P and 110R) Banazi Black grape varieties which were 5 years old were used in the study conducted in Turkey, Malatya, Yesilyurt district, Konak town. The grapevines in the farmer vineyards, grown in dry conditions, were established in the form of blocks, serpene cultivation was applied on the stocks, and cane training was made. The photochemical characteristics of the dried grape belonging to the rootstocks used in 5 year-old Banazi Black grape which were ungrafted (5 and 40 years old ungrafted) and grafted with 41B, 99R, 1103P, 110R rootstocks were investigated in this study. It was determined from the analyzed phenolic compounds that gallic acid was 4.10-14.43 mg/kg; catechin was 146.87-306.87 mg/kg, rutin was 38.96-78.15 mg/kg, naringin was 22.54-50.97 mg/kg, phloridzin was 40.03-107.72 mg/kg, and quercetin was 0.47-1.67 mg/kg. Total antioxidant capacity in total phenolic substance rootstocks was determined to be 0.19-0.22 mg trolox equivalent/g; gallic acid/g was 7.51-21.66 mg. The amount of resveratrol was between 0.19-1.30 mg/kg among the rootstocks. The differences between the rootstocks were statistically significant in terms of phenolic compounds. 40 year-old ungrafted vinestock took a higher value when compared to the other rootstocks in terms of phenolic compounds.
Keywords: *dried grape, grafted-ungrafted, antioxidant content, phenolic content, Malatya*

Introduction

Turkey is an important vine cultivation center in the world. According to the data of TÜİK [Turkish Statistical Institute] (2016), 4,000,000 tones grapes, including 1,990,604 tones table, 1,536,269 tones dried, and 472,534 tones wine grapes, were grown totally in the 4,352,269 da. area in Turkey (Anonymous, 2017).

The farming of cultivated grapevine (*Vitis vinifera* L.) has been conducted in Turkey since 6000-5000 B.C. (Doğer, 2004). Grapevine is an important cultivated plant in Turkey as it is economical in terms of grape yield and a genetic material with its abundant varieties. (Celik, 1998; Celik et al., 1998). This abundance of the grapevine gene potential creates an important source for both the rehabilitation works and the production of the local types that have economic importance.

Grapevine has always kept its value among the agricultural products with its richness in terms of gene potential, its economic importance, and its significance in human nutrition and health. Generally, there are water, sugars, organic acids, phenol

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compounds, pectic substances, aroma substances, nitrogenous substances, enzymes, vitamins and minerals in the compound of grapes (Fidan and Yavas, 1986; Canbas, 2003; Jackson, 2003).

The species and varieties of grapevines are the foremost plant species that are rich in phenolic compounds (Lohachoompol et al., 2004). These compounds are synthesized structurally in all organs of the grapevine and their proportions in fruit and seeds are affected by environmental conditions (Ough and Amerine, 1988).

Phenolic components which are defined as the secondary metabolism products of the plants are the primary natural compounds standing out with their antioxidant characteristics in terms of human health. According to scientific studies, phenolic compounds have antiallergic, antiinflammatory, antidiabetic, antimicrobial, antipathogenic, antiviral and antithrombotic effect (Knekt et al.,1996; Le Marchand et al., 2000; MacDougall, 2002; Aras, 2006; Kafkas et al., 2006).

One of the distinguishing characters of the colorful (red, black) grapes is phenolic compounds. It has been determined in the researches that colorful types are richer than white types in terms of phenolic substances (Cemeroğlu, 2004).

Just as each grape variety has a different tendency in terms of soil and climate, their response to the American grape-vine rootstock on which they are grafted may be different. For this reason, the most appropriate chemical component a grape type can reach is directly related to the soil structure and climate of the territory it is cultivated in and the rootstock on which it is grafted (Amerine et al., 1972; Jackson, 2000; Ribéreau-Gayon, et al., 2000; Canbas, 2006; Kelebek, 2009; Rolle et al., 2010).

There are some studies on the influence of the local grape varieties in Turkey, the vineyards that they are cultivated in, and the climatic conditions on the phenolic compounds of the grapes, yet these studies are not enough. However, vine cultivation activities are distributed in different geographical regions in our country which have different climatic characteristics. The fact that these regions have different characteristics in terms of climatic and soil conditions will make a difference in terms of phenolic compounds which directly affect the quality factor of our local grape varieties. For this reason, new studies which will deal with our local grape varieties the regions in which they are cultivated are needed.

Banazi Black grape is cultivated throughout Malatya, but it has specifically adapted to Konak town (Banazi), Yesilyurt and Akcadağ districts which are located in 1000-1300 m above sea-level (Koc et al., 2015).

People prefer ungrafted cultivation, since they think grapes have a specific taste, flavor, and color. However, the fact that the vineyards in the region are limy and waterless led people to use rootstocks resistant to lime and aridness. The use of rootstocks is important because it provides earliness in Banazi Black grape (Koc et al., 2015).

It was determined in the studies that phenolic compound concentration varies by grape variety, the environment it is cultivated and environmental factors. This study aims to determine some photochemical characteristics of Banazi Black grape, which has adapted to Malatya Yesilyurt ecology and is cultivated with or without grafting, within the scope of the significance of rootstock-variety relationship in vine cultivation.

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Materials and methods

Material

Our study, the analyses of which were carried out in Malatya Apricot Research Institute research and application laboratories, was conducted in 2014 (38°16′53.31” N - 38°17′13.38” E, at 1290 m altitude) (*Figure 1*).

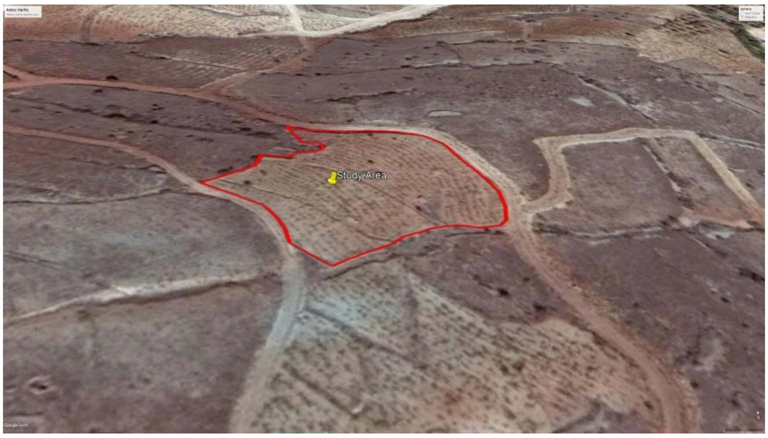

Figure 1. *The research center where the experiments were conducted*

The materials of the study are ungrafted 5 and 40-year old Banazi Black grape and 5 year-old Banazi Black grape variety grafted on 41B, 99R, 1103P, 110R American grape-vine rootstocks.

The study was conducted in the farmer vineyard, where serpene cultivation was applied on the stocks shaped like blocks in dry conditions with 2×2 distances and cane training was made, in the control of Malatya Apricot Research Institute personnel in Malatya Konak (Upper Banazi) area which is 1290 m above sea-level.

Banazi Black grape which ripens between September 10th and 20th is a seed bearing, local variety which is dried in clusters and put on market naturally. Grapes are blue-black, globular and moderate-sized. The fact that it is dried with its cluster provides a perceptual superiority for the consumer. The clusters are conical, medium (250 g-350 g) and dense (*Figure 2*).

Figure 2. *Fresh and dried Banazi Black grape fruit*

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It is not subjected to any chemical process during drying process. Harvest starts when the first withering appears on the grapes of the cluster on the stock. The clusters that are harvested are subjected to drying process either directly on the soil or on the cloths laid on the soil (*Figure 1*) Drying process lasts between 5-9 days (Koc et al., 2015).

Geographic position of the research location

Yesilyurt, which is one of the two central districts of Malatya with its surface area of 1013 km², is surrounded by Battalgazi on the east, Akcadağ and Doğansehir on the west, Celikhan and Adıyaman on the south, Yazıhan on the north. Yesilyurt district where the vineyard used in the test is located is on a transit area between Southeastern Anatolia Region terrestrial precipitation and Mediterranean Region marine precipitation, and between Eastern Anatolia Region terrestrial precipitation and Central Anatolia Region precipitation (Anonymous, 2015).

Soil characteristics of the research location

Earth sample taken from 0-30 cm depth in the vineyard was analyzed in Malatya Apricot Research Analysis Laboratory (*Table 1*).

Table 1. *Soil analysis values of the test field*

Saturation (%)	**pH**	**Total salt (%)**	**Lime (%)**	**Organic matter (%)**	**EC (µS/cm)**	**P (kg/da)**	**K (kg/da)**
51.70	7.75	0.0228	37.90	1.33	0.69	8.48	77.89

It can be seen on *Table 1* that since saturation value is 51.70, vineyard where the research was conducted has “Clay-Loam” soil. It can be seen that the test field is saltless with a value of 0.02%, is very limy with 37.90% lime ratio, has low organic substance with 1.33% ratio, and has an adequate structure with 8.48 kd/da phosphor and 77.89 kg/da potassium. It was determined that it had loose-alkali structure with 7.75 pH and its electrical conductivity EC (mS/cm) value was 0.690. As a consequence of the soil analysis 300-400 g sulfur and 15-20 kg burnt farm manure were applied on each stock during the early spring.

Climatic characteristics of the research location

Some important meteorological data belonging to Malatya-Yesilyurt district ecology where the research was conducted are given on *Table 2* and *Table 3* (Anonymous, 2015).

The ripening of the clusters belonging to the stocks coincided with September in which the average temperature was 22.7 °C during the time when the research was conducted in 2014. During the harvest season in this year total precipitation was 53.8 mm for September, monthly average relative humidity was 32.6%, and minimum temperature was 8.3 °C for September, 2014 (*Table 3*).

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***Table 2.** Some climatic data belonging to the research location*

Months	Monthly minimum temperature (°C)				Monthly average temperature (°C)			
	2011	**2012**	**2013**	**2014**	**2011**	**2012**	**2013**	**2014**
January	-5.2	-10.0	-9.6	-4.3	2.4	0.6	0.9	4.1
February	-6.1	-10.0	-4.5	-7.2	3.1	-1.5	-	-
March	-1.7	-6.5	-3.9	-3.4	8.4	3.4	8.8	10.4
April	-0.9	3.6	5.7	1.4	12.2	14.9	14.9	15.4
May	6.6	-	9.5	9.9	17.0	-	19.5	19.6
June	12.9	11.0	12.6	12.4	23.5	25.6	24.5	23.9
July	16.6	13.9	16.1	19.0	29.0	28.7	27.2	30.3
August	15.1	16.4	-	18.8	28.0	28.3	-	30.8
September	13.2	13.8	11.2	8.3	23.1	-	21.8	22.7
October	4.5	8.3	4.0	3.7	14.9	16.6	14.5	15.3
November	-6.1	1.7	1.7	0.5	4.7	10.7	10.6	7.3
December	-4.5	-2.4	-	-0.4	1.7	3.5	-	6.4

*(-) No value was assigned by the Directorate of Meteorology
**(0.0) There is no precipitation. Monthly maximum temperature was not given by the Directorate of Meteorology

***Table 3.** Some climatic data belonging to the research location*

Months	Monthly total precipitation (mm)				Monthly relative humidity (%)			
	2011	**2012**	**2013**	**2014**	**2011**	**2012**	**2013**	**2014**
January	23.3	39.0	62.4	29.4	66.4	-	-	-
February	63.3	51.0	52.2	28.5	-	-	-	-
March	18.4	22.8	20.1	40.2	46.1	51.3	50.5	48.0
April	95.8	27.1	39.6	50.1	60.2	-	45.7	44.9
May	42.9	-	773	36.0	53.1	-	46.2	40.5
June	10.0	15.5	10.8	18.3	35.8	28.3	26.7	28.8
July	5.5	1.5	0.0	0.0	26.9	23.6	23.2	19.7
August	8.4	0.2	-	0.7	25.9	24.7	-	19.5
September	5.2	0.0	17.1	53.8	29.3	-	29.6	32.6
October	6.2	58.0	12.9	92.2	39.9	-	34.0	56.4
November	40.8	64.6	25.1	56.0	-	-	-	-
December	42.8	84.9	-	31.6	67.4	-	-	-

*(-) No value was assigned by the Directorate of Meteorology
**(0.0) There is no precipitation. Monthly maximum temperature was not given by the Directorate of Meteorology

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Method

The test was planned to be in three repetitions with 6 stocks for each one in the vineyard where the research was conducted, which was shaped like blocks.

Sampling

Sample grape clusters for each stock and each of the three repetitions were taken when the first withering started on the clusters of the stocks (Koc et al., 2015), the color and taste specific to the variety formed and the dry matter amount reached to 23-24 Brix value, and these samples were left for drying on cloth shelves.

The grapes on the dried clusters belonging to the stock in each repetition were plucked and ground in blenders. These samples were put into glass jars to be analyzed later and kept in -20 °C.

Extraction of the phenolic components

2.5 g was taken from the Banazi Black grape sample which was dried, ground, and homogenized, and it was put into a falcon tube of 50 ml. 15 ml of methanol:water:HCI (70:29, 9:0.1) solvent mixture which was prepared previously was added on the sample and it was homogenized. The samples which were kept in dark for 12 h were centrifuged at 9000 rpm and transferred to a new supernatant falcon tube. 15 ml solvent mixture was added on the residue and it was mixed and centrifuged again. The supernate was added to the previous one. Extraction process was repeated for the third time. After about 45 ml of extract was obtained, the same extract was evaporated in a vacuum evaporator in inert environment until dryness. Rhe residue was dissolved with 3 ml methanol:water (50:50), syringed, filtered through 0.45 µm cellulosic filter, and the final filtrate was used in the determination of phenolic components, total phenolic substance amount and total antioxidant capacity (Milan et al., 2011).

Determination of phenolic components with HPLC

First, RT (retention time) in HPLC system and wavelength were determined for each of the 17 different phenolic standard compounds (*Table 4*). Then, mixture standard including all phenolic compounds was prepared and its calibration chart was drawn. Two different solvent mixtures were used as the mobile phase.

Mobile Phase A: Water: Acetic Acid (95.5%: 4.5%), Mobile Phase B: Acetonitrile

Table 4. *Gradient program applied on HPLC system*

Analysis time (mn)	Solvent (B)	Flow rate (ml/mn)	Temperature (°C)	Wavelength (λ)
0.01	0	1	30	280,290,355,310,329,370
7	5	1	30	280,290,355,310,329,370
12	15	1	30	280,290,355,310,329,370
20	40	1	30	280,290,355,310,329,370
25	100	1	30	280,290,355,310,329,370
30	100	1	30	280,290,355,310,329,370
40	5	1	30	280,290,355,310,329,370

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Determination of total phenolic substance amount

The content of total phenolic compound was determined according to Folin-Cicalteu spectrophotometric method developed by Slinkard and Singleton (1977) in the study. “Folin & Ciocaltue” method was used to determine total phenolic substance amount and total phenolic substance values were measured in terms of gallic acid. After 50 µl was taken from the extracts and 950 µl water was added on it, 1 ml of Folin-Ciocaltue solution was added and it was kept waiting for 3 min. After this period, 1 mL was made 3 mL with 2% Na_2CO_3 solution, it was kept waiting for 10 min, and reaction was balanced. Absorbances of the colored solutions were measured to be 760 nm in terms of wavelength.

Determination of total antioxidant capacity

Two tests, DPPH Radical Scavenging Power Test and TEAC (Trolox Equivalent Antioxidant Capacity) were carried out.

DPPH radical scavenging power test

Radical scavenging power (RSP) measurement was carried out according to formula 1 suggested by Yen et al. (2000). The samples which were prepared in 1.10-4 M DPPH ethanol were left for incubation at ambient temperature with extracts obtained from grape samples in 0.1 ml of different concentrations and 2.9 ml of DPPH solution that were put into in spectrophotometer tubs. During the control, ethanol was put instead of the sample. The absorbance of the tubes was read against 517 nm after 30 min of incubation. RSG values were calculated according to the following formula (*Eq. 1*).

$$RSG = [1 - \frac{A_{Ö:30}}{A_{K:30}}] \times 100 \quad (1)$$

$A_{Ö:30}$: For example, absorbance in the 30^{th} min of $A_{K:30}$: Control

TEAC (Trolox Equivalent Antioxidant Capacity) test

7 mM ABTS (2,2-Azinobis 3-ethylbenzothiazoline 6-sulfonic acid) was mixed with 2.45 mM potassium bisulfate and it was kept waiting in a dark environment for about 12-16 h. This solution was purified with 0.700±0.01 absorbance in 734 nm wavelength with sodium acetate (pH 4.5) buffer in spectrophotometer, 2.98 mL of the solution was blended in 20 µL fruit extract, and the absorbance was measured in 734 nm wavelength in spectrophotometer ten minutes later. The obtained absorbance values were calculated with Trolox (10-100 µmol/L) standard slope chart and it was stated as µmol Trolox equivalent/g fruit (Özgen et al. 2006).

Statistical analysis

SPSS packaged software was used in determining the influence of rootstocks on photochemical characteristics and the differences between the averages were stated with Duncan multiple comparison test. 3 repetitions and the averages of 10 clusters taken from 6 vinestocks in each repetition were used for the characteristics measured in the study. Statistical materiality level was considered as $P < 0.05$.

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Results

Findings concerning the change of phenolic components

Quercetin, catechin, gallic acid, rutin, naringin and phloridzin dehydrate in Banazi black were found to be in identifiable levels. As can be seen in *Table 5,* the examined phenolic compounds were statistically significant in comparison with the rootstocks. Average gallic acid was determined to be at the lowest amount in 99R rootstock (4.10 mg/kg) and at the highest amount in 40-year old ungrafted vinestock (14.43 mg/kg). Catechin was at the lowest amount in 110R rootstock (146.87 mg/kg) and at the highest amount in 40 year-old ungrafted vinestock (306.87 mg/kg). Rutin was at the lowest amount in 99R rootstock and at the highest amount in 40-year old ungrafted vinestock (38.96-78.15 mg/kg). Naringin was determined in ungrafted young and 40 year-old vinestocks with 22.54-50.97 mg/kg, Phloridzin was in 99R and 40 year-old ungrafted vinestocks with 40.03-107.72 mg/kg, and Quercetin was in 110R and 40 year-old ungrafted vinestocks with 0.47-1.67 mg/kg.

Table 5. *Some phenolic compounds in detectable levels in the study*

Rootstocks	Gallic acid mg/kg dry	Cateşin mg/kg dry	Rutin mg/kg dry	Naringin mg/kg dry	Phloridzin mg/kg dry	Quercetin mg/kg dry
Ungrafted (young)	5.164bc	212.60b	75.44ab	22.54b	50.22c	0.65c
Ungrafted (old)	14.43a	306.87a	78.15a	50.97a	107.72a	1.67a
1103P	8.939b	187.54bc	75.48ab	35.91b	79.43b	1.51a
110R	5.304bc	146.87c	53.75c	32.68b	51.45c	0.47c
99R	4.107c	184.44bc	38.96d	29.93b	40.03d	1.08b
41B	6.697b	173.23bc	67.78b	34.01b	75.56b	1.04b
Significance	0.592	0.001	0.008	0.000	0.001	0.021

*$P < 0.05$ level of significance. Different letters show that the difference between the averages is significant according to Duncan test

Taking the average values into consideration, 99R and 110R rootstocks, known to be late season variety, were at lower levels when compared to the other rootstocks in terms of the determined phenolic compounds. The values of the clusters obtained from 40 year-old ungrafted vinestocks were recorded to be higher than the other rootstocks' values.

Total antioxidant capacity (TAC)

There was no statistically important difference among the rootstocks in terms of free radical (DPPH) removal activity in determining total antioxidant capacity (TAC) in the study. TAC values were determined to be 0.19-0.22 mg /g between the rootstocks (*Table 6*).

It can be seen from *Table 6* that there were statistically significant between the rootstocks in determining TEAC (trolox equivalent antioxidant capacity). While the lowest average value was obtained from 99R rootstock, the highest value was determined to be in the clusters obtained from 40 year-old ungrafted vinestocks (0.17-0.70 mg/g). The values in other rootstocks were young ungrafted vinestock 0.18 mg/g, 1103P 0.59 mg/g, 110R 0.49 mg/g, 41B 0.50 mg/g.

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Table 6. *Resveratrol, total antioxidant capacity and phenolic substance amount*

Rootstocks	Total antioxidant capacity (mg trolox equivalent /g antioxidant capacity)		Total fenolik (mg gallik asit/g dry)	Resveratrol (mg/kg dry)
	DPPH	ABTS		
Ungrafted (young)	0.19b	0.18c	11.60bc	1.30a
Ungrafted (old)	0.20ab	0.70a	21.37a	0.32b
1103P	0.21a	0.59ab	14.73b	0.49b
110R	0.20ab	0.49b	17.08ab	0.71ab
99R	0.22a	0.17c	7.51c	0.49b
41B	0.19b	0.50b	21.66a	0.19b
Significance	0.255	0.017	0.159	0.021

*$P < 0.05$ level of significance. Different letters show that the difference between the averages is significant according to Duncan test

Bozan et al. (2008) revealed in their study, in which they investigated the antiradical effects of different grape varieties growing in our country, that different grape varieties removed DPPH radical in different proportions with the effect of their different phenolic contents.

Total phenolic substance amount (TPSA)

Average total phenolic substance amount (TPSA) in dried Banazi Black grape variety changed between 7.51-21.66 mg gallic acid/g. The differences between the rootstocks in terms of TPSA were considered statistically significant in our study. The lowest value was obtained from 99R rootstock and the highest value was obtained from 41B rootstock and 40 year-old ungrafted vinestocks (21.66-21.37 mg gallic acid/g). TPSA in the clusters obtained from the other rootstocks was 110R 17.08 mg/g, 1103P 14.73 mg gallic acid/g and TPSA in ungrafted young vinestock was 11.60 mg gallic acid/g.

Özden and Vardin (2009) determined in their study that Chardonnay (3170 mg/kg) variety had high phenolic substance amount, followed by 2376 mg/kg Merlot 9 variety, and total phenolic contents of Cabernet sauvignon and Syrah varieties were 1968 and 1805 mg/kg, respectively.

Alphonse Lavallèe and Horoz Black grape varieties were determined to be the ones with the highest production of total phenolic substances, 3.084 and 2.832 mg/g, respectively (Cetin, 2012).

In the study on ripe grapes belonging to 12 wine grape varieties, Singleton (1966) determined that total phenolic compound amount in terms of gallic acid was 3770 mg/kg as an average value (when it is converted into the unit in our study, the result is 3.77 mg/g). The researcher also determined that phenolic compound amounts were different according to species and varieties.

Resveratrol

It was determined as a result of the analyses that Banazi Black grape contained the phenolic compound named in many studies as resveratrol, which has antioxidant, antimutagenic, antiinflammatory, anticarcinogenetic and chemoprotective effects.

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Resveratrol amount changed in 0.19-1.30 mg/kg between the rootstocks. The lowest average value was determined in the grapes obtained from 41B rootstock and the highest value was determined in the grapes obtained from young ungrafted vinestocks. For 40 year-old ungrafted ones, it was determined to be 0.32 mg/kg, 1103P 0.49 mg/kg, 110R 0.71 mg/kg and 99R 0.49 mg/kg (*Table 6*).

The flavonoid contents of 500 µl fruit extract of Banazi Black are 1523.07 µg catechin, 591.79 µg rutin and 1.6661 µg resveratrol (Özşahin, 2010).

Discussion

In this study, the changes of young-old and rootstock-variety performances in the levels of phenolic compounds, total antioxidant capacity (TAC), total phenolic substance amount (TPSA) and resveratrol after Banazi Black grape variety, which has an important position in Malatya as seed-bearing grape for drying with its gene potential, was grown on different rootstocks in its original ecology were investigated within the scope of the importance of rootstock-variety relationship in vine cultivation practices.

Because common and interconverted units are not used to evaluate phenolic compound results in literature studies, the comparison and discussion of the results have become a problem. For this reason, the obtained values were compared to the studies using similar or interconvertible units (mg/kg, mg/100g, mg/g, g/kg etc.).

The differences among the rootstocks in terms of TPMS amount were considered statistically significant in our study. According to the averages, the lowest value was obtained from Banazi Black grape grafted on 99R rootstock, the highest value was obtained from Banazi Black grape grafted on 41B rootstock and 40 year-old ungrafted vinestock (21.66-21.37 mg gallic acid/g). Taking the worldwide known wine grape varieties such as Cabernet sauvignon (1968 mg/kg), Merlot (2376 mgkg), Syrah (1805 mg/kg) and edible variety Alphonse Lavallée (3466 mg/kg) into consideration (Singleton, 1966; Núñez et al., 2004; Özden and Vardin, 2009; Kelebek, 2009) 7.51-21.66 mg gallic acid/g values (when the units were converted, Cabernet sauvignon 1.968 mg/g, Merlot 2.376 mg/g, Syrah 1.805 mg/g, Alphonse Lavallée 3.466 mg/g) obtained in our study were over these limits.

Aras (2006) determined that TPSA content changed between 2.88-3.42 mg/g for red grapes and 1.87-2.22 mg/g for white varieties. Karakaya et al. (2001) reported that total phenolic substance amount was 3.99 mg/g for dried grapes and 2.21 mg/g for red grapes. When these data are compared to the ones in our study, we can say that Banazi Black grape contains a very high amount of TPSA.

According to studies, while TPSA concentrations of black grapes are 1800 mg/kg on average, this value is 405 mg/kg for white varieties (Frankel et al., 1995; Shahidi and Naczk, 1995).

Cultivation conditions, rootstocks that are used, grape variety, and extraction and analytical methods that are used are considered to have an effect on the values found by the researchers.

Free radical (DPPH) removal activity was determined to be 0.19-0.22 mg/g (41B and 99R) in determining total antioxidant capacity (TAC) in the study. There was no statistically important difference among the rootstocks.

The differences among the rootstocks were considered statistically significant in determining TEAC (trolox equivalent antioxidant capacity). In terms of the averages,

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the lowest value was obtained from Banazi Black grape grafted on 99R rootstock and the highest value was in ungrafted old vinestock (0.17-0.70 mg/g).

Banazi group in the samples of 25µl concentrations had radical cleaning effect in a very significant proportion when compared to the other samples in a study comparing free radical cleaning activities of the grape extracts (Özsahin, 2010).

When Bozan et al. (2008) investigated the antiradical effects of different grape varieties cultivated in our country, they revealed that different grape varieties removed DPPH radical in different proportions because of their different phenolic contents.

According to a study, while CAT (catalase antioxidant enzyme) activities of Sultani seedless grape variety grafted on 1103P, 110R and 41B rootstocks in low salt concentration increased, CAT activities of Sultani seedless grape variety grafted on 1616C and 1103P rootstocks in high salt concentration increased. SOD (super oxide dismutase) activities of Sultani seedless grape variety grafted on 1103P, 110R and 99R rootstocks in high salt application increased. On the contrary, SOD activities of Sultani seedless grape variety grafted on 140Ru and 41 B rootstocks decreased. In low salt application SOD activity on 1616C 140Ru, 110R and 41B rootstocks decreased. According to the control, AP (ascorbate peroxidase) activities of Sultani seedless grape variety grafted on 110R, 99R and 41B rootstocks in low salt application increased while AP activities of 1616C and 140Ru rootstocks decreased. AP activity of Sultani seedless grape variety grafted on 110R rootstock in high salt application increased (Sahin, 2009).

This study on Sultani seedless grape grafted on different rootstocks show that the effect of rootstocks on antioxidative enzyme activities is significant. According to our study, the statistical differences in TEAC determination on six rootstocks belonging to a single variety resulted from the rootstocks.

Quercetin, catechin, gallic acid, rutin, naringin and phloridzin dihydrate were found in detectable levels in the extracts of dried Banazi Black grape in our study. Gallic acid was determined to be at the lowest amount in Banazi Black grape grafted on 99R rootstock (4.10 mg/kg) and at the highest amount in ungrafted old vinestock (14.43 mg/kg). Catechin was at the lowest amount in Banazi Black grape grafted on 110R rootstock (146.87 mg/kg) and at the highest amount in ungrafted old vinestock (306.87 mg/kg). Rutin was at the lowest amount in Banazi Black grape grafted on 99R rootstock and at the highest amount in ungrafted old vinestock (38.96-78.15 mg/kg). Naringin was determined in ungrafted young and old vinestocks with 22.54-50.97 mg/kg, Phloridzin was in Banazi Black grape grafted on 99R and ungrafted old vinetocks with 40.03-107.72 mg/kg, and Quercetin was in Banazi Black grape grafted on 110R and ungrafted old vinestocks with 0.47-1.67 mg/kg. According to a study, the amount of total phenols increase with the ripening of leaves; old leaves on the same vinestock contain more phenolic compounds than young leaves (Medeghini et al., 1992). According to our study, the phenolic compound amount obtained from ungrafted old vinestocks is higher than the phenolic compound amount obtained from Banazi Black vinestocks produced as young grafted and ungrafted vinestocks.

The differences among the detected phenolic compounds were considered statistically significant in the rootstocks. Taking these values into consideration, it is considered that the rootstocks used in our study are effective on phenolic compound amount.

In another study done by Aydınlık (2012), phenolic substance analyses of the grape molasses (pekmez) samples produced in Niğde were conducted with the method of HPLC. It was found out that grape molasses (pekmez) samples contained gallic acid,

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catechin, caffeic acid, epicatechin, p-coumaric acid and ferulic acid and their concentrations were 47.94±2.58, 148.69±11.17, 20.7±2.08, 101.25±5.8, 12.24±1.65 and 18.26±2.58 mg/kg, respectively.

Resveratrol amount changed between grafted and ungrafted rootstocks in 0.19-1.30 mg/kg. the lowest value was obtained from Banazi Black grape grafted on 41B rootstock and the highest value was obtained from the young vinestock without rootstocks.

Flavonoid and resveratrol contents of Banazi Black grape extracts were determined to be 1980.00±2.88 mg/g for catechin, 769.33±2.33 mg/g for rutin, 2.16±0.16 mg/g for resveratrol (Özsahin, 2010). These values show that Banazi Black grape has a rich phenolic substance source in harmony with our study.

Bartolomeo (1996) and Zhao (1999) reported that the fundamental phenolic content of the grape consisted of resveratrol, catechin ve epicatechin. In addition, the studies showed that grape was a rich phenolic compound source and 46-69% of these phenolic compounds was in the seed, 12-50% was in the skin, 8% or less was in the pulp.

Phenolic substance amount of the grapes change depending on the variety, ripeness, environmental conditions such as climate and soil and applied cultural processes (Ribéreau-Gayon et al., 2000). However, the quality and quantity of phenolic compounds of grapes depend on grape variety. While the existence of certain phenolic substances and the proportions of their amounts to each other depend on the characteristics of species and varieties genetically controlled, total phenolic substance amount or the sort of phenols in the compound depend on environmental factors (Singleton and Esau, 1969).

The content of phenolic compounds changes based on the soil conditions in which the vinestock is cultivated and cultural applications. The soils with high sodium content and pH reduce phenolic substance amount in the seed, peel and stem (Quintana and Gomez, 1989), and irrigation applications increase total phenol amount in leaves and offshoots (Madero et al., 1978). Besides, the form of cultivation, rootstock and herbicide applications affect the content of phenolic substances in the vinestock and grape (Bezhanishvili et al., 1982; Smart and Smith, 1988). In line with our study, these studies conducted by other researchers show that rootstocks are effective in terms of phenolic compound contents.

In addition to the reasons reported in the previous studies, the difference in the values of our study may result from the fact that dried Banazi Black grape is ground and analyzed with its peel, pulp and seed and from the rootstocks that are used. As a matter of fact, according to a study, the 38% of the total phenol content of red and white grape varieties is seeds, 36% is peel, 20% is stem and 6% is pulp (Flanzy et al., 1972).

The researchers who determined that pure and hybrid rootstocks used in modern vine cultivation has different effects based on the content of grape juice, wine quality and coloration (Ruhl, 1991; Bisson, 1992; Kaserer and Schofll, 1994) reported that sugars, amino acids, pH and K level in the content of anthocyanin, acidity and grape peel color changed with grafting, and acidity increased excessively with the increase in productivity and affected wine quality.

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Conclusion

One of the oldest forms of conserving fruits and vegetables is drying, its importance still continues. Drying grapes for preserving is the easiest and most economical way of conservation that has been implemented for years (Dokuzoğuz, 1972).

Because both edible grapes and various products produced from them are rich in phenolic compounds and they have bioactive characteristics for human health, grape, especially black grape, is a food product that must be a part of our nutrition.

When the data in this study is taken into consideration, the fact that grafting is effective on Banazi Black grape type produced with or without grafting, with regard to Resveratrol, Total Phenolic Substance amount, Total Antioxidant Capacity and some Phenolic Compounds, becomes more important. The obtained results are expected to provide an insight into the studies that will be conducted in future, since the current study is the first study conducted on dried grape of "Banazı".

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IMPACT OF SOIL CONDITIONERS AND WEATHER ON LAWN COMPACTNESS

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Abstract. The aim of this paper is to determine the effect of soil conditioners and one mineral fertilizer on turf, as well as their interaction with meteorological conditions in maintaining of lawn compactness. The field experiment was conducted in the experimental facility between 2013 and 2015. The following grass species were used in the experiment: *Lolium perenne*, *Festuca rubra*, and *Poa pratensis*. Another experimental factor tested in the research was soil conditioners. Between 2013 and 2015 an assessment of grass compactness based on the methods described by Domański (1998) was carried out. This assessment was conducted in three seasons: spring, summer, and autumn. Grass species tested in the experiment formed turf of different compactness, varying throughout seasons of the year and responding strongly to weather conditions. In drier periods red fescue had better turf, contrary to smooth meadow grass and perennial ryegrass, both of which had better turf in more humid conditions. Out of all the types of fertilizer Humus Active Papka affected turf compactness in the most favourable way although the response of individual grass species was not the same. From a practical point of view out of the grass species tested in the experiment *Poa pratensis* treated with Eko-Użyźniacz responded the most favourably.
Keywords: *compactness, turf quality, red fescue, smooth meadow, grass perennial ryegrass*

Introduction

Architects dealing with spatial planning pay more and more attention to green areas, with lawns, relatively inexpensive to set up, being their important element. Lawns have multiple functions, from environmental, through sanitary-hygienic, to recreational and aesthetic (Czeluściński et al., 2017).

The increase in the standard of living in Poland and other countries is accompanied by the awareness of the importance of green areas and the need for an attractive and healthy environment (Watsson et al., 1992). Lawns are common in parks, gardens, streets, squares, and recreational areas (Ignatieva et al., 2015; Bertoncini et al., 2012; Stewart at al., 2009). Dense and uniform turf is the most important element of lawn functional value (Domański 1998; Grabowski et al., 1999; Harkot and Czarnecki, 2001; Harkot et al., 2006; Jankowski et al., 2010, 2011a; Prończuk et al., 1997). According to Jankowski et al. (2011d, 2012a) as well as Rutkowska and Pawluśkiewicz (1996) turf quality largely depends on its treatment and maintenance, on habitat conditions, but also on the proper selection of grass species and varieties used to set up a lawn (Wheeler et al., 2017).

Grass is commonly used for lawns, regardless of their type. Some 160 grass species can be found in Poland (Prończuk and Żurek, 2008). Yet, with such a huge number only 16 species are suitable for lawns. *Festuca rubra* (red fescue), *Poa pratensis* (smooth

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meadow grass), and *Lolium perenne* (perennial ryegrass) are grass species most common on lawns used extensively (Jankowski et al., 2012b).

To a large extent lawn quality depends on frequent watering, which in turn affects maintenance costs (Jankowski et al., 2011a; Jankowski et al., 2011b; Jankowski et al., 2011c; Jankowski et al., 2011d; Jankowski et al., 2011e). The use of modern watering systems is not always a good solution. Usually those systems are too expensive and not every owner can afford such an investment (Jankowski et al., 2012a).

With the growing interest in the use of environmentally friendly products, numerous soil conditioners have been approved for the use in green areas, fulfilling the role of fertilizer, or chemicals used in plant protection. Continuous progress in the studies on soil conditioners shows their competitiveness in relation to conventional solutions.

The aim of this paper is to determine the effect of soil conditioners and one mineral fertilizer on turf, as well as their interaction with meteorological conditions in maintaining of lawn compactness. The study deals with practical aspects of the impact of soil conditioners on the compactness value of individual species of lawn grass. In addition, the research aims at getting information about the extent to which soil conditioners can replace mineral fertilizer without degrading functional value of the lawn.

Material and methods

Set up in 2012 the field experiment was conducted in the experimental facility of the University of Natural Sciences and Humanities in Siedlce (52°12'N, 22°28'E) between 2013 and 2015. The research was carried out as a mini-plot experiment, in the split plot design with three replications and the plot area of 1 m^2. The following grass species were used in the experiment (factor B): *Lolium perenne* -variety Info, *Festuca rubra* -variety Nil, and *Poa pratensis* -variety Alicja. They were sown on their own, each of them at the rate of 28 g/m^2. Another experimental factor tested in the research was soil conditioners (factor A)

Soil conditioners used in the experiment improve soil properties, according to the Institute of Soil Science and Plant Cultivation (IUNG) in Puławy. The composition of the soil conditioners used in the research is presented in *Table 1*.

The UGmax soil conditioner is an extract from compost, containing macronutrients (N, P, K, Mg, Na) and micronutrients (Mn). It also contains lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas*, yeast, and *Actinomycetes*. The micro-organisms in the conditioner have a capacity of processing organic and natural fertilizers into compost and humus. These processes are conducive not only to the production of humus but also to improving soil structure, which in turn has a positive effect on water balance in the soil. In addition, the UGmax soil conditioner increases disease resistance, keeping plants healthy, but it also stimulates the development of the root system and supports the biological reduction of molecular nitrogen.

Humus Active Papka contains macronutrients (N, P, K, Ca, Mg), trace elements (Mn, Fe, Zn, Cu), and active humus with useful microorganisms. According to the manufacturer Humus Active, among other beneficial effects, positively affects plant health as well as soil structure, and releases nutrients not readily available to plants.

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Table 1. *Composition of soil conditioners applied in the experiment.*

Soil conditioner	Macronutrients ($g \cdot kg^{-1}$)					Micronutrients ($mg \cdot kg^{-1}$)						Microorganism and others
	N	**P**	**K**	**Ca**	**Mg**	**Na**	**Mn**	**Fe**	**Zn**	**Cu**	**Mo**	
Substral (S)	220	21.8	83		12.06		12	50	12.5	12.5	1	-
Humus Astive Papka (HAP)	0.2	1.3	4.6	3.0	0.5	-	15	500	3	1	-	Active humus with useful microorganisms
Eko-Użyźniacz (EU)	0.6	0.3	0.7	-	-	-	-	-	-	-	-	Endo micorhizza, fungi, bacteria, enzymes of earthworms
UGmax (UG)	1.2	0.2	2.9	-	0.1	0.2	0.3	-	-	-	-	lactic acid bacteria, photosynthetic bacteria, Azotobacter, Pseudomonas, yeast, Actinomycetes

Eko-Użyźniacz is extracted from bovine vermicompost and contains the main macronutrients (N, P, K), micro-organisms, and enzymes related to metabolism of earthworms. This soil conditioner stimulates biological life in soil degraded chemically, mechanically, or biologically, and increases plant resistance to different stress factors.

Soil conditioners were applied annually in spring (mid May) in the following doses: UGmax – 25 ml $\cdot$ m^{-2} (0.6 l in 250 l of water), Eko-Użyźniacz - 100 ml $\cdot$ m^{-2} (10 l in 100 l of water), and Humus Active Papka - 250 ml $\cdot$ m^{-2} (0.2 l for 10 l of water). In turn, Substral, a slow release fertilizer used on lawns, was used in the quantity of 20 g $\cdot$ m^{-2}.

At the end of each growing season between 2013 and 2015 an assessment of grass based on the methods described by Domański (1998) was carried out. Among other things its compactness was assessed in accordance with the methodology by Prończuk and Żurek (2008), using a 9-point rating scale in which 1 stands for no aesthetic value; 3 for unattractive appearance; 5 for average; 7 for attractive; 9 for very attractive. This assessment was conducted in three seasons: spring, summer, and autumn. In each year of the research spring assessment was made around May 20, summer assessment around 20 August, and autumn assessment around October 10.

The experiment was set up on the soil developed from loamy sand, belonging to anthropogenic soils of the culture-earth order, and of the type of hortisole (Systematics of Polish Soils). Chemical analysis showed that the soil was alkaline, with high content of magnesium and phosphorus, and low potassium content. The test results were evaluated statistically with the analysis of variance. Tukey's test ($P \leq 0.5$) was used to find significantly different means of the effects of experimental factors and their

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interaction. Based on lawn appearance ratings, standard deviation and coefficient of variation were calculated for separate seasons, years, and soil conditioners.

Weather conditions

Meteorological data between 2013 and 2015 were obtained from the Hydrological and Meteorological Station in Siedlce. The average air temperature during the experiment was very similar to the long-term average temperature *(Table 2)*. In each year of the research the highest temperature was in July (19.3 on average) and August (19.0), while the lowest temperature, taking into account only the growing season, was in April (an average of 8.5) and October (an average of 8.2).

Table 2. *Average air temperature (°C) and precipitation (mm) in individual months of the growing seasons.*

Year	Month							
	Apr.	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**	**Means**
Temperature (°C)								
2013	7.5	15.3	17.7	18.8	18.3	11.4	9.6	14.1
2014	9.7	13.7	15.1	20.5	17.8	13.7	8.4	14.1
2015	8.2	12.3	16.5	18.7	21.0	14.5	6.5	14.0
Means	8.5	13.8	16.4	19.3	19.0	13.2	8.2	14.1
Long-term means	8.5	14.0	17.4	19.8	18.9	13.2	7.9	14.2
Rainfall (mm)								
2013	57.6	145.8	111.9	49.1	44.1	86.6	18.0	73.3
2014	39.5	79.5	74.2	37.5	105.7	26.3	3.0	52.2
2015	30.0	100.2	43.3	62.6	11.9	77.1	39.0	52.0
Means	42.4	108.5	76.5	49.7	53.9	63.3	20.0	59.2
Long-term means	33.0	52.0	52.0	65.0	56.0	48.0	28.0	47.7

A meteorological element with major temporal fluctuations is rainfall (Skowera and Puła, 2004). By analyzing its amount in different months of the experiment it was found that the lowest average monthly precipitation was in 2015 (52.0 mm), but still it was higher by 2.3 mm than the long-term average. In the growing season of 2013 the average monthly precipitation of 73.3 mm was the highest. May 2013 was a month in which rainfall was the highest of all months during the experiment (145.8 mm). The lowest rainfall was recorded in October 2014 (3.0 mm) and August 2015 (11.9 mm).

In order to determine the temporal variation of meteorological elements and their effects on vegetation, Sielianinov's hydrothermal coefficient (Bac et al., 1993) was calculated on the basis of monthly rainfall (P) and the monthly total air temperature (Σt), using the formula: $K = P/0.1\ \Sigma t$ (Skowera and Puła, 2004).

In assessing temperature and moisture conditions ten-scale classification of Sielianinov's hydrothermal coefficient (K) was used, which, according to Skowera and Puła (2004), has the following ranges:

$K \leq 0.4$ extremely dry (ss);
$0.4 < K \leq 0.7$ very dry (bs);
$0.7 < K \leq 1.0$ dry (s);

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$1.0 < K \le 1.3$ quite dry (ds);
$1.3 < K \le 1.6$ optimal (o);
$1.6 < K \le 2.0$ quite wet (dw);
$2.0 < K \le 2.5$ wet (in);
$2.5 < K \le 3.0$ very wet (bw);
$K > 3.0$ extremely wet (sw).

The values of Sielianinov’s hydrothermal coefficient for individual months of the experiment are shown in *Table 3*. It was assumed that the conditions were extreme when coefficient values were below 0.7 and above 2.5 (Skowera and Puła, 2004).

Table 3. *Sielianinov’s hydrothermal coefficient (K) during the growing season.*

Years	Month						
	Apr.	**May**	**June**	**July**	**Aug.**	**Sept.**	**Oct.**
2013	2.56 (bw)	3.07 (sw)	2.11 (w)	0.84 (s)	0.78 (s)	2.53 (bw)	0.60 (bs)
2014	1.36 (o)	1.87 (dw)	1.64 (dw)	0.59 (bs)	1.92 (dw)	0.64 (bs)	0.12 (ss)
2015	1.22 (ds)	2.63 (bw)	0.87 (s)	1.08 (ds)	0.18 (ss)	1.46 (o)	1.94 (dw)

Optimal temperature and moisture conditions were only in April 2014 and in September 2015. In the remaining months of all the growing seasons the weather conditions were not as favourable, varying from extremely dry in August 2015 to extremely wet in May 2013. Throughout the experiment the best conditions occurred at the beginning of each growing season. It can be concluded that the most difficult period for plants was in 2015, when, apart from May and the end of the growing season, the weather conditions ranged from quite dry to extremely dry.

Results and discussion

According to different authors (Prończuk et al., 1997; Harkot and Czarnecki, 2001; Jankowski et al., 2011a), dense and uniform turf has a big impact on the functional value of a lawn. Lawn appearance is one of the most important features determining its compactness. Rutkowska and Pawluśkiewicz (1996) point out that turf compactness is influenced by many factors, such as environmental conditions, the kind and frequency of maintenance treatment, the appropriate selection of grass species and varieties, and the way in which the lawn is used.

Table 4. *Assessment of turf compactness (9-point scale) in the spring from 2013 to 2015.*

Year (C)	Species (B)	Fertilizers (A)				$\bar{x}$
		(S)	**(EU)**	**(HAP)**	**(UG)**	
2013	Smooth meadow grass	8.8	9	8.9	8.6	8.8
	Perennial ryegrass	7	6.9	7.8	6.8	7.1
	Red fescue	8.7	8.9	9	9	8.9
2014	Smooth meadow grass	8.9	8	8.1	8.6	8.4
	Perennial ryegrass	8.7	8.3	7.6	8.9	8.4
	Red fescue	8.3	6.2	5.9	8.8	7.3
2015	Smooth meadow grass	7.8	8.3	8.1	8.1	8.1

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	Perennial ryegrass	9	8.7	8.9	7.9	8.6
	Red fescue	8.7	8.9	9	9	8.9
		Species effect - means				
Smooth meadow grass		8.5	8.4	8.4	8.4	8.4
Perennial ryegrass		8.2	8	8.1	7.8	8
Red fescue		8.6	8	8	8.9	8.4
		Fertilizer effect - means				
		8.4	8.1	8.4	8.4	8.3
		Average annual assessment				
2013		8.2	8.3	8.6	8.1	8.3
2014		8.6	7.5	7.2	8.8	8
2015		8.5	8.6	8.7	8.3	8.5

$LSD_{0.05:}$ A=NS; B=NS; C=NS; A/B=NS; B/A=NS; A/C=1.04; C/A=0.93; B/C=0.81.; C/B=0.81.

Turfs of the lawns studied in this experiment were diverse both in terms of the species tested and the fertilizer applied. Their compactness changed throughout years and seasons of research. Assessment of the lawn turfs showed that their quality in the spring (*Table 4*) varied throughout the experiment, and it was different for different species of grass. In the spring of the first and third year red fescue had the best turf (8.9^{o}) in comparison with the other species. In turn, in the second year (2014) the compactness of this grass was the worst of all of them (7.3°). These differences were statistically significant.

Changes in the compactness of red fescue turf were related to the weather. Red fescue is one of those species that reach optimum development in habitats with variable water conditions and often even in dry places. Therefore, in 2013 and 2015, in which years there were a lot of dry periods (*Tables 2 and 3),* this grass formed a good quality turf. Similarly, in the studies of Grabowski et al. (1999) and Jankowski et al. (2011c) seeding mixtures with a high proportion of red fescue formed the best turf. According to Golińska (2002) high turf-forming potential of red fescue is a specific and important feature.

Throughout the research in the spring seasons (*Table 4)* both smooth meadow grass and red fescue had the best turf, both at the level of 8.4°, as the average of three years between 2013 and 2015. According to Martyniak and Prończuk (2003), chewings fescue has rhizomes of varying length underground, and they form very strong and dense turf. Therefore, it is an excellent grass with good compactness turf on grassland, lawns, and other areas. Additionally, comparing different species with different types of fertilizer applied, it was found that the best turf in the spring was on the plot with red fescue treated with UGmax (8.9°). The worst lawn (7.8°) was on the plot with perennial ryegrass, also treated with UGmax. In the case of smooth meadow grass lawn compactness did not vary much (from 8.4 to 8.5°), no matter what type of fertilizer was used. In the studies of Kitczak et al. (2000) smooth meadow grass had a better turf than a mixture with perennial ryegrass.

Assessing the effect of fertilisers in the spring it was found that for all of the grass species turf compactness was similar, no matter whether Substral, Humus Active Papka, or UGmax was applied, being at the same level of 8.4°. Eko-Użyźniacz was an exception causing slightly weaker lawn compactness (8.1°)

Assessing the effect of different forms of fertilizer in different spring seasons, turf of the best compactness was in the spring of 2015 (from 8.3 to 8.7°). That year the best

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grass was on the plots with Humus Active Papka (8.7°) and Eko-Użyźniacz (8.6°). Similarly, in 2013 these forms of fertilizer ensured the best lawns (8.6 and 8.3°, respectively). In 2014, which was the wettest year (*Tables 2* and *3*), turf on plots treated with Humus Active Papka and Eko-Użyźniacz was the worst (7.2 and 7.5°, respectively), but it was best of all as a result of UGmax (8.8°) and Substral (8.6°) application. Statistical analysis showed a significant interaction between the year of the research and the form of fertilizer in their impacts on turf compactness.

Table 5. *Assessment of turf compactness (9-point scale) in the summer from 2013 to 2015.*

Year (C)	Species (B)	Fertilizers (A)				$\bar{x}$
		(S)	(EU)	(HAP)	(UG)	
2013	Smooth meadow grass	8.8	7.9	9	9	8.7
	Perennial ryegrass	8.9	7.6	7.5	9	8.3
	Red fescue	3.3	7.9	8.4	3.8	5.9
2014	Smooth meadow grass	9	9	8.9	8.8	8.9
	Perennial ryegrass	8.7	9	8.8	8.9	8.9
	Red fescue	9	8.8	8.9	8.9	8.9
2015	Smooth meadow grass	5.9	8.2	6.1	7.3	6.9
	Perennial ryegrass	9	5.6	8.4	7.8	7.7
	Red fescue	2.8	4.2	3.1	3.9	3.5
		Species effect - means				
Smooth meadow grass		7.9	8.4	8	8.4	8.2
Perennial ryegrass		8.9	7.4	8.2	8.5	8.3
Red fescue		5	7	6.8	5.5	6.1
		Fertilizer effect - means				
		7.3	7.6	7.7	7.5	7.5
		Average annual assessment				
2013		7	7.8	8.3	7.3	7.6
2014		8.9	8.9	8.9	8.9	8.9
2015		5.9	6	5.9	6.3	6

$LSD_{0.05:}$ A=NS; B=1.44.; C=1.44; A/B=NS; B/A=NS; A/C=NS; C/A=NS; B/C=2.25; C/B=2.25.

In the summer *(Table 5)* turf compactness was slightly worse than in the spring time, and to a greater extent it was dependent on weather conditions (*Tables 2 and 3*). The best possible degree of turf compactness for each species of grasses was in 2014 (on average 8.9° for each species). The spring that year was rainy (*Table 3*), with hydrothermal coefficient indicating optimal conditions in April, but quite wet in May and June. Similarly, during the summer that year weather conditions were much more favourable for grass than in the other two years of the research. For all the grass species the highest average compactness rating (8.9°) was also in 2014, when, on average, lawns treated with different types of fertilizer had the same turf compactness. Comparing all the species throughout the experiment, in summer seasons perennial ryegrass formed the best lawn (on average 8.3°), and with references to the forms of fertilizer, Substral was the most effective (8. 9°). Red fescue had the worst lawn in summer (an average of 6.1°), and with regard to fertilizer types, plots with Substral (5°) and UGmax (5.5°) had turf of the lowest compactness. Statistical analysis revealed significant differences in the degree of turf compactness between red fescue and the other two species of grasses. The interaction between the form of fertilizer and grass

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species in their effect on the turf compactness turned out to be statistically significant. Comparing the effect of the forms of fertilizer on all grass species during summer in all experimental years, the application of Humus Active Papka (7.7°) resulted in the best turf, while the effect of Substral (7.3°) was the worst. Differences between the impacts of both of them were not statistically significant.

Table 6. *Assessment of turf compactness (using 9-point scale) in the autumn from 2013 to 2015.*

Year (C)	Species (B)	Fertilizers (A)				$\bar{x}$
		(S)	(EU)	(HAP)	(UG)	
2013	Smooth meadow grass	9	8.9	8.6	8.9	8.9
	Perennial ryegrass	8.5	9	9	8.9	8.9
	Red fescue	8.9	9	9	8.7	8.9
2014	Smooth meadow grass	8.2	8	8.1	7.9	8.1
	Perennial ryegrass	5.6	6.9	6.8	5.8	6.3
	Red fescue	8.7	9	9	8.9	8.9
2015	Smooth meadow grass	8	8.7	8.8	7.8	8.3
	Perennial ryegrass	8.9	8.7	9	8.9	8.9
	Red fescue	8.8	8.9	9	9	8.9
		Species effect - means				
Smooth meadow grass		8.4	8.5	8.5	8.2	8.4
Perennial ryegrass		7.7	8.2	8.3	7.9	8
Red fescue		8.8	9	9	8.9	8.9
		Fertilizer effect - means				
		8.3	8.6	8.6	8.4	8.5
		Average annual assessment				
2013		8.8	9	8.9	8.8	8.9
2014		7.5	8	8	7.4	7.8
2015		8.6	8.8	8.9	8.6	8.7

$LSD_{0.05:}$ A=NS; B=0.67; C=0.67; A/B=NS; B/A=NS; A/C=NS; C/A=NS; B/C=0.64; C/B=0.64.

The turfs in autumn seasons *(Table 6*) were to a large extent a reflection of meteorological conditions (*Tables 2* and *3).* During this period the most adverse weather conditions for grass development were in 2014 (*Table 3*), when September was very dry and October extremely dry. In effect, turf compactness was the worst (an average of 7.8°) although red fescue that year was in good condition (on average 8.9°), while much worse lawns were those with perennial ryegrass (on average 6.3°). Those differences were statistically significant. In the other two years (2013 and 2015), when water conditions were by far better, turf was at its best (from 8 to 9°). The experiment showed significant differences in turf compactness between grass species and between autumn seasons. As an average for all grass species, the best turf was in 2013 and 2015. In addition, the best turf was obtained as a result of the application of Eko-Użyźniacz (9.0 in 2013 and 8.8° in 2015) and Humus Active Papka (8.9° in both years). In autumn seasons red fescue had the best turf (from 8.8 to 9.0°), and with regard to the type of fertilizer, in the same seasons both Eko-Użyźniacz (9 in 2013 and 8.8 in 2015) and Humus Active Papka (8.9° in 2013 and 2015) application resulted in a very good lawn.

Out of the grass species in the autumn perennial ryegrass had the worst turf (on average 8.0°). Statistical analysis showed that the interaction between the grass species

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and the year of research in their effect on lawn compactness was statistically significant. In assessing the effects of fertilizer types on all grass species in each year, it was found that in the autumn Eko-Użyźniacz and Humus Active Papka increased turf compactness in autumn seasons the most (8.6°).

Table 7. *Fertilizer effect on turf compactness (9-point scale). Means within species, years, and season.*

Species (B)	Fertilizers (A)				$\bar{x}$
	(S)	(EU)	(HAP)	(UG)	
Smooth meadow grass	8.3	8.4	8.3	8.3	8.3
Perennial ryegrass	8.3	7.9	8.2	8.1	8.1
Red fescue	7.5	8	7.9	7.8	7.8
Fertilizer effect - means					
	8	8.1	8.2	8.1	8.1
Year (C)	**Average annual assessment**				
2013	8	8.4	8.6	8.1	8.3
2014	8.3	8.1	8	8.4	8.2
2015	7.7	7.8	7.8	7.7	7.7
Average assessment in seasons (D)					
spring	8.4	8.1	8.4	8.4	8.3
summer	7.3	7.6	7.7	7.5	7.5
autumn	8.3	8.6	8.6	8.4	8.5

$LSD_{0.05}$: A=NS; B=0.41; C=0.49; D=0.33

By comparing average grass species ratings in the experiment as a whole *(Table 7)* it turned out that smooth meadow grass had the best turf (8.3°) and red fescue the worst (7.8°). Differences in the degree of turf compactness between grass species were statistically significant, especially between smooth meadow grass and red fescue. Grabowski et al. (1999) pointed out that the advantage of smooth meadow grass is its durability and high turf-making potential. In Poland in this regard this species easily competes with perennial ryegrass. Out of the types of fertilizer Humus Active Papka application resulted in the best turf (8.2°) in the experiment, although differences in its compactness on plots with different forms of fertilizer were small and not statistically significant. With regard to the seasons it was found that generally the best turf was in the autumn (8.5°) and spring (8.3°) and the worst in the summer (7.5°). Differences in the degree of its compactness between the summer, the spring, and the autumn were not statistically significant. Whatever the time of the year, in subsequent years of research the average assessment rating systematically deteriorated from 8.3° in 2013 to 7.7° in 2015, and these differences were statistically significant.

Table 8. *Standard deviation and coefficient of variation in assessing of turf compactness*

Species	*Min. rating*	Max. rating	Means values	Standard deviation	Coefficient of variation
Smooth meadow grass	*5.9*	9	8.33	0.737	8.84
Perennial ryegrass	*5.6*	9	8.10	1.052	12.98
Red fescue	*2.8*	9	2.06	2.062	26.48

Coefficient of variation: 0-20% - small variability, 20 – 40% - medium variability, 40-60% - large variability, > 60% - very large variability

By analyzing the durability of compactness ratings in relation to particular grass species *(Table 8)* it turned out that coefficient of variation for smooth meadow grass and perennial ryegrass was the most favourable, indicating moderate variability. For red fescue the coefficient of variation of compactness rating was above 20%, indicating medium variability. Therefore, to install a lawn, smooth meadow grass both on its own and in mixtures, should be recommended first of all.

Conclusions

1. Grass species tested in the experiment formed turf of different compactness, varying throughout seasons of the year and responding strongly to weather conditions. In drier periods red fescue had better turf, contrary to smooth meadow grass and perennial ryegrass, both of which had better turf in more humid conditions.
2. Out of all the types of fertilizer Humus Active Papka affected turf compactness in the most favourable way although the response of individual grass species was not the same.
3. Out of all the grass species smooth meadow grass and red fescue had the best turf on plots where Humus Active Papka was applied, and perennial ryegrass had the best turf on plots with Substral. In the subsequent years of research there was a deterioration of lawn compactness, with a weakening effect of all types of fertilizer, in particular in the case of Eko-Użyźniacz and Humus Active Papka.
4. From a practical point of view out of the grass species tested in the experiment P*oa pratensis* treated with Eko-Użyźniacz responded the most favourably.

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VOLATILE COMPONENT COMPOSITION OF *BALLOTA NIGRA* SUBSP. *ANATOLICA* AT DIFFERENT VEGETATION PERIODS IN ÇAMLICA PROVINCE OF KÜTAHYA CITY, TURKEY

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Abstract. The volatile component composition of leaves and flowers for *Ballota nigra* subsp. *anatolica* P.H. Davis that were collected between 2015 and 2016 from Çamlıca provice of Kütahya city during three different vegetation periods as pre-bloom, bloom and post flowering were determined by gas chromatography mass spectroscopy (GC-MS) after solid phase micro extraction (SPME). 59 different volatile oil components were determined of *Ballota nigra* subsp. *anatolica*. As main components, Hexenal (17.60%), germacrene D (6.70%) and β-caryophyllene (8.80%) were found during pre-blooming period, also Hexenal (21.16%), germacrene D (7.77%) and β-caryophyllene (10.03%) in blooming period and Hexenal (15.88%), germacrene D (7.60%) and β-caryophyllene (9.48%) in post-flowering period.
Keywords: *SPME, black horehound, volatile component, Lamiaceae*

Introduction

The research on the essential oil content of medicinal plants is quite important both in scientific and economic aspects. The increase in microorganism resistance against all known antibiotics has made it necessary to investigate plant sources and their effects on cells. There are also essences, most of which constitute essential oils in medicines as well as some substances such as cellulose, pectin, sugar and it is known that these substances have an important pharmacological effect (Kilic, 2005)

Lamiaceae family, one of the greatest families that can be grown almost anywhere without discrimination of habitat type and height, is the most spread especially in the Mediterranean Basin (Watson and Dallwitz, 1978). This family has 200 genus and approximately 3200 species and family members represented with 45 genus and more than 546 species in Turkey are important in pharmacology and perfumery industry because they contain essential and aromatic oil (Davis, 1982, Baytop, 1991). Lamiaceae has a rate of endemism of 44.2% in Turkey (Baser, 1993).

The genus *Ballota* L., which is a member of the Lamiaceae family that constitutes the material of this study, has about 35 taxa around the world (Patzak, 1958). It is represented with 12 species and 8 subspecies in Turkey (Guner, 2012). *Ballota* L. is a well-known genus in Europe due to its spasmolytic and sedative effects (Garnier et al., 1961). It is used in folk medicine for cough, asthma, headache, nausea, hemorrhoids, wound and burn treatment (Baytop, 1984).

The Black horehound (*Ballota nigra*) bearing only simple hairs. Stems erect, 50-100 cm, simple or branched above. Cauline leaves ovate-orbicular to ovate, 25-70 x 20-50 mm, acute, crenate dentate, truncate or rounded at base, distinctly petiolate. Inflorescence long, lax below. Floral leaves 1-2 x dense verticillasters. Bracteoles subulate, usually shorter than calyx tube. Calyx 7-10 mm, tubular obconical, dilated above into usually 5, very short to attenuate teeth that are porrect to patent or recurved,

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mucronate or aristate. Corolla purple or pink, 9-14 mm; upper lip concave, emarginate or erose (Davis, 1982; Anonymous, 2016).

In this study, volatile components of leaf and flower of *Ballota nigra* subsp. *anatolica* P.H. Davis, that belong to three different vegetation periods including prebloom, bloom and post-flowering were determined by SPME (solid-based microextraction method).

Materials and Methods

The materials of this study which was conducted in 2015 and 2016 are leaves and flower samples of *Ballota nigra* subsp. *anatolica* P.H. Davis that were collected in 3 different periods as pre-flowering term, flowering term and post-flowering from Çamlıca province (39° 43′ N, 29° 91′ E) of Kütahya city which is situated in the inner part of western Turkey.

The leaves and flower samples that were collected were put into paper packages and transferred to the laboratory in the same day without kept waited and exposed to sunlight. After the plant materials collected were dried at room temperature (25°C), flower and leaf samples were subjected to solid phase microextraction (SPME). 2 g of samples were placed into a 10 mL vial. After incubation for 30 min at 60°C, SPME fibre was pushed through the headspace of a sample vial to adsorbed the volatiles, and then inserted directly into the injection port of the GC-MS (Shimadzu 2010 Plus GC-MS with the capillary column, Restek Rxi®-5Sil MS 30 m x 0.25 mm, 0.25 µm) at a temperature of 250°C for desorption (5 min) of the adsorbed volatile compounds for analysis. Identification of constituents was carried out with the help of retention times of standard substances by composition of mass spectra with the data given in the Wiley, NIST Tutor, FFNSC libraries.

Results

59 different volatile components were specified by gas chromatography mass spectroscopy (GC-MS) after solid phase micro extraction (SPME) for *Ballota nigra* subsp. *anatolica*. The results are given in *Table 1*. Hexenal, germacrene D and β-caryophyllene were specified as the main components of *Ballota nigra* subsp. *anatolica*. Hexenal (17.60%), germacrene D (6.70%), and β-caryophyllene (8.80%) in the prebloom period; Hexenal (21.16%), germacrene D (7.77%) and β-caryophyllene (10.03%) in the bloom period, Hexenal (15.88%), germacrene D (7.60%), β-caryophyllene (9.48%) in post flowering period were identified (*Table 1*). It was observed that there was an increase in the proportion of volatile components during the bloom period.

Table 1. *Volatile components of Ballota nigra subsp. anatolica P.H. Davis according to different vegetation periods*

	Retention Time	Components	Pre-bloom (%)	Bloom (%)	Post-flowering (%)
1	1.401	Isobutanal	0.17	0.70	-
2	1.438	2-Methylpropenal	-	0.87	-
3	1.549	2-Ethylbutanal	1.52	1.14	1.20
4	1.581	2-Methylfuran	1.10	0.96	1.30
5	1.635	3-Methylfuran	0.93	0.48	0.70

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	Retention Time	Components	Pre-bloom (%)	Bloom (%)	Post-flowering (%)
6	1.910	2-Butenal	1.17	1.39	0.64
7	1.949	3-Methylbutanal	1.41	1.24	0.55
8	2.031	2-Methylbutanal	1.33	1.38	0.25
9	2.246	1-Ethylcyclopropanol	1.70	1.30	0.75
10	2.372	Pentanal	6.16	6.93	6.32
11	2.970	(E)-3-Penten-2-one	1.30	0.85	1.11
12	3.025	3-Methylenepentane	0.27	0.33	0.20
13	3.260	(E)-2-Pentenal	1.57	0.90	0.59
14	3.544	2-methyl-1-Penten-3-one	0.55	0.40	-
15	3.584	Pentanol	-	0.38	0.32
16	3.642	2-Penten-1-ol	0.44	0.26	0.25
17	3.870	3-Methyl-2-butenal	0.45	0.25	0.55
18	4.037	2-Acetylfuran	0.55	0.22	0.25
19	4.225	Hexanal	17.60	21.16	15.88
20	5.692	(E)-2-Hexenal	3.06	3.00	3.20
21	5.855	cis-3-Hexene-1-ol	5.65	6.83	5.52
22	6.184	trans-2-Hexen-1-ol	0.70	0.42	0.44
23	6.302	Hexanol	0.67	0.56	2.20
24	6.572	2,6-dimethyl-Pyridine	0.50	0.51	-
25	6.808	2-Heptanone	0.50	0.50	0.71
26	7.137	(Z)-4-Heptenal	-	0.20	0.40
27	7.218	Heptanal	0.39	0.53	0.20
28	7.548	2,4-Hexadienal	0.54	0.68	0.50
29	8.007	α-thujene	1.48	0.14	1.66
30	8.238	α-pinene	1.20	0.31	1.85
31	8.321	2,7- Dimethyloxepine	0.17	0.18	-
32	9.088	6-methyl-2-Heptanone	0.30	0.20	0.30
33	9.193	(Z)-2-Heptenal	1.50	1.30	1.25
34	9.353	Benzaldehyde	1.74	1.52	2.12
35	9.974	Cyclopentane, 1,2,4-trimethyl-, (1α)	0.71	0.64	0.33
36	10.281	1-octen-3-ol	0.80	0.20	1.34
37	10.446	2-pentyl-Furan	2.40	2.34	2.23
38	10.732	(E,E)-2,4-Heptadienal,	1.87	1.46	2.82
39	10.881	<ethyl->Hexanol	0.82	0.28	0.63
40	10.960	Octanal	0.90	0.73	0.51
41	11.297	(E,E)-2,4-Heptadienal	1.27	1.44	1.94
42	11.765	p-Cymene	1.53	1.67	1.49
43	11.913	Limonene	4.67	5.24	5.70
44	12.312	3-Octen-2-one	1.96	1.90	2.51
45	12.496	Bicyclo[6.1.0]nona-5,8-dien-4-one	1.14	1.04	1.13
46	12.794	2-ethyl-6-meth-1,5-heptadiene	0.30	0.25	0.23
47	13.076	2 Octenal	0.34	0.39	0.41
48	13.545	3,5-octadien-2-one	1.13	0.85	1.49
49	14.393	1-Undecene	2.45	2.73	2.65
50	14.886	Nonanal	1.39	1.07	1.59
51	18.263	Methyl salicylate	0.82	0.60	0.85
52	18.787	Decanal	0.30	0.19	0.30
53	21.967	3-hexadecene	0.40	0.25	0.60
54	24.845	Copaene	1.23	0.21	1.57
55	25.118	β-bourbonene	0.41	0.37	0.54
56	26.328	Germacrene D	6.70	7.77	7.60
57	26.076	β-caryophyllene	8.80	10.03	9.48

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	Retention Time	Components	Pre-bloom (%)	Bloom (%)	Post-flowering (%)
58	28.390	Cyclopropanecarboxylic acid 2,2-dimeth	0.82	0.17	0.65
59	31.585	Caryophyllene oxide	0.22	0.16	0.20

Discussion and Conclusions

Bader et al. (2003) studied the volatile oil components of *Ballota nigra* ssp. *foetida*, *B. undulata* and *B. saxatilis*. β-caryophyllene (25.1%) and germacrene D (24.2%) were identified as the main components of *Ballota nigra* ssp. *foetida*. Morteza-Semnani et al. (2007) determined 42 components, specifying caryophyllene oxide (7.9%), epi-α-muurolol (6.6%), δ-cadinene (6.5%), and α-cadinol (6.3%) as the main components. The main components differ according to our study. Kazemizadeh et al. (2009) found the germacrene D (18.1%), nerolidol epoxyacetate (15.4%), sclareol oxide (12.1%), linalyl acetate (11.5%), and β-caryophyllene (10.5%) as the main components in *B. nigra* subsp. *anatolica*. Vukovic et al. (2009) determined 115 components in *Ballota nigra* in Serbia. They reported that β-caryophyllene, germacrene D, and α–humulene in the leaves and stalks, p-vinylguiacol (9.24%), borneol (7.51%), myrtenol (7.13%), trans-pinocarveol (5.22%), pinocarvone (4.37%), 2-methyl-3-phenylpropanal (4.32%), and p-cymen-8-ol (4.30%) in the roots as the main components.

Fraternale et al. (2009) identified 37 components in *Ballota nigra*. They found β-caryophyllene (20.0%), germacrene D (18.0%), and caryophyllene oxide (15.0%) as the main components. Fraternale and Ricci (2014) have specified β-caryophyllene (22.6% and 21.8%), caryophyllene oxide (18.0% and 20.5%) and germacrene D (16.5 and 13.1%) as the main components in *Ballota nigra* L. ssp. *foetida*. In our study, germacrene D (7.77%) and β-Caryophyllene were determined as the main components, sharing similarities to other works. Unlike other studies, hexenal component were identified.

Jamzad et al. (2013) reported 12 different components including β-pinene (39.0%) and α-pinene (34.5%). However, 59 different components were found, specifying Hexenal (21.1%), germacrene D (7.77%) and β-caryophyllene (10.03%) as the main components. The main components in our work differ from those of Jamzad et al. (2013).

As conclusion; *Ballota* taxa are consumed in South Anatolian regions against cough, asthma, headache, nausea and hemorrhoids, and they are used externally in wound and burn treatment. Studies should be increased for people to consume and use consciously. It is considered that local people will be informed, random collection of plants and economic losses to be incurred due to false information will be prevented, and collection of plants will be performed more consciously. Further studies related to volatile components and possibilities of their usage within the scope of antibacterial, antimicrobial and deterrent etc. should be investigated.

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SPRINGS (THE KRKONOŠE MOUNTAINS NATIONAL PARK, CZECH REPUBLIC): SPECIES DIVERSITY IN RELATION TO ENVIRONMENTAL FACTORS

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Abstract. The springs are unique ecotones that integrate ecological characteristics and human impacts associated with both underground and surface water as well as terrestrial ecosystems. A clear definition of the management of springs is a precursor to their effective protection and restoration. Species diversity of springs areas in the eastern part of the Krkonoše Mts. was investigated in eight various localities. By a cover-abundance scale species richness (plants and mosses) and habitat type (phytocenological survey) were evaluated. Statistical analyses were used to compare springs by main gradients with respect to environment determinants (Principal component analysis with environmental supplementary variables, Simpson's dominance index and Shannon's diversity index). A total of 59 vascular plants, 9 mosses and 4 communities were found, including 8 endangered taxa of them. The springs of Modry brook and Renner brook had the highest species diversity and the largest number of vascular plants were observed on the headwater area of Upa River. The fewest endangered species were determined on the springs of Suchy brook and Javori brook. The resulting species richness is a function of the combination of altitude and exposure, where the highest species richness values were recorded in the highest located subalpine headwater areas.
Keywords: *wetland vegetation; Montia fontana; plant communities; altitude; subalpine spring*

Introduction

Springs are ecosystems in which groundwater reaches the Earth's surface either at or near the land-atmosphere or land-water interface. At their sources (orifices, points of emergence), the physical geomorphic template allows some springs to support numerous microhabitats and large arrays of aquatic, wetland, and terrestrial plant and animal species. Yet, springs ecosystems, however, differ significantly from other aquatic, wetlands and riparian ecosystems (Stevens et al., 2005). They fundamentally contribute to the circulation of nutrients, soil formation, climate regulation, carbon accumulation, nutrient and water retention (Sorooshian and Whitaker, 2003). As wetland habitats are one of the most threatened in all of Europe, detailed knowledge of relationships between springs vegetation and environmental conditions is, among others, highly important for applications in nature conservation. This study is intended to fill a large gap in the knowledge of the springs vegetation diversity in Krkonoše high-mountain regions and its ecological determinants.

The Krkonoše was proclaimed as a government-protected area for water accumulation because of its significance as a headwater area. The collecting area of

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underground waters is shallow and has a relatively low springs discharge. The springs water maintains a year-around temperature between 4 - 6 °C, so the headwater areas do not freeze (Stursa et al., 2012). Thanks to various geologic and ecologic conditions, headwater areas include a wide range of locations (Danks and Williams, 1991).

Twelve springs types are recognized, not including paleo-springs (Springer and Stevens, 2009). In a recent study biology of springs widely used simple division into three basic types (according to the method of seepage of ground water and the intensity of its velocity: Limnocrene; Helocrene; Rheocrene), as aptly describes the nature of springs (Cantonati et al., 2006). This differentiation of communities is primarily conditioned by the ground water mode; water chemical composition, temperature, nutrition content, springs discharge, water flow rate and slope angle. The most significant species diversity is influenced by mineral richness and the reaction of springs water (Tahvanainen et al., 2002; Nekola, 2004; Novakova, 2007).

Earlier vegetation studies carried out by Czech and Poland researchers were mostly performed using the dominance approach (see Chumanova-Vavrova et al., 2015; Vacek et al., 2017). Moreover, springs have been investigated only marginally in these studies. Studies of springs vegetation that focus on the overall species composition of the vegetation are very rare in Czech Republic. A vegetation study from Czechoslovakia (Hadac, 1983) and the syntaxonomic evaluation of spring communities with *Montia fontana* (Hadac and Vana, 1971) represent the exceptions. In the present study, we describe the diversity of Krkonoše high-mountain springs with respect to major environmental factors measured directly in the field.

Vegetation of springs is widespread throughout Europe, and is more frequent at higher altitudes with humid climates. Spring represent azonal habitat, sometimes referred to as hydrological sub-climax (Hinterlang, 1992). The *Montio-Cardaminetea* class comprises vegetation developing in springs with cold and well oxygenated water. Cold water with low nutrient content reduces vascular plant productivity, while leading to increasing bryophyte cover. Enhanced input of nutrients can therefore quickly change the structure of springs vegetation to the benefit of vascular plants (Hajkova et al., 2006). Variability of this class is relatively large and therefore may occur in the springs ecologically distinct from foothills to alpine levels in deciduous and coniferous woods and on open sites (Zechmeister and Mucina, 1994). With increasing distance from a springs, flow rates and oxygenation decrease and springs vegetation is replaced by other communities, typically mire and fen vegetation of the class *Scheuchzerio palustris-Caricetea nigrae* (Økland et al., 2001).

By describing vegetation diversity, we examine the major ecological determinants of vegetation variation springs in a biogeographical context. Another objective is to evaluate the floristic elements in ecologically diverse springs vegetation types in the Krkonoše Mountains National Park, Czech Republic.

Materials and methods

Study area

A field survey was conducted at the The Krkonoše Mountains National Park (Czech Republic, official abbreviations KRNAP – KPN). Krkonoše is home to the highest mountains in the Czech Republic, but that is also true throughout Central Europe to the north of the Alps. In fact, the mountains form the northernmost montane border of Central Europe, stretching in length just over 50° of northern latitude, whilst their slopes

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protrude above the alpine tree line. Consequently, the Krkonoše Mountains represent a mighty and natural barrier on the perimeter of large open plains in Germany and Poland. They measure approximately 35 km in length, with their main ridges and valleys arranged in a direction from northwest to southeast. This significantly affects all the geographical, climatic and biological features of these European medium-sized mountains and their surroundings. Therefore, Krkonoše Mountains are a truly important area for geobiodiversity in Central Europe.

Vegetation samples were taken from representative springs, which we found in Krkonoše Mountains during our extensive research in vegetation seasons 2015 –2016. Eight springs were chosen:

L1 – Zeleny brook 50°42′42.704′′N 15°40′26.351′′E | L2 – Modry brook 50°43'21.799"N 15°41'23.273"E | L3 – Javori brook 50°39′30.148′′N 15°44′27.238′′E | L4 – Weber brook 50°40′33.614′′N 15°44′19.803′′E | L5 – Renner brook 50°43′45.719′′N 15°49′29.460′′E | L6 – Max brook 50°39′26.506′′N 15°50′58.014′′E | L7 – Suchy brook 50°39′45.649′′N 15°51′26.606′′E | L8 – Upa River (so called ′Deliquescent Rock′) 50°40′1.685′′N 15°47′321.445′′E – for more characteristics, see *Table 1* and *Fig. 1*.

Table 1. *Summary of different springs at different sites of the The Krkonoše Mountains National Park (Czech Republic)*

	Exposure	Soil	Altitude (m a.s.l.)	Slope (°)	Coverage E_1 (%)	Coverage E_0 (%)	Springs type
L1	East	Podsol	1 375	12	70	40	Limnocrene
L2	South	Podsol	1 350	39	70	50	Helocrene
L3	North	Podsol	1 220	14	15	90	Helocrene
L4	Northwest	Podsol	1 110	9,5	60	50	Helocrene
L5	West	Podsol	1 080	11,3	70	50	Helocrene
L6	West	Cambisol	965	54	90	15	Rheocrene
L7	West	Cambisol	940	10,5	60	20	Helocrene
L8	East	Podsol	625	69	80	5	Rheocrene

The phyto-sociological part was worked out with the help of the Zürich Montpellier School of Phytosociology and included analysis, synthesis and identifying vegetation. Sixteen phyto-sociological relevés were taken (Rosenthal, 2003). The vegetation plot size was delimited in such a way as to represent full floristic composition of the phyto-coenosis. It varied from 3.75 to 12 m^2 depending on plant density and the homogeneity of vegetation cover (Chytry and Otypkova, 2003).

Cover of vascular plants and bryophytes was estimated using the seven-grade Braun-Blanquet scale (van der Maarel, 1979). Vegetation and environmental data were simultaneously collected in submontane and lowland springs and from subalpine tall-form vegetation. The slope of the sample plot was subjectively estimated. Altitude and coordinates were measured by GPS Garmin Oregon 400t (WGS 84 system) with altimeter calibrated by current atmospheric pressure. Other environmental factors (soil types, aspect) were noted for each relevé.

The nomenclature of vascular plants follows Danihelka et al. (2012) and the bryophytes nomenclature follows Kucera et al. (2012). The vascular plant species were classified into five groups according to their threat status by Grulich (2012).

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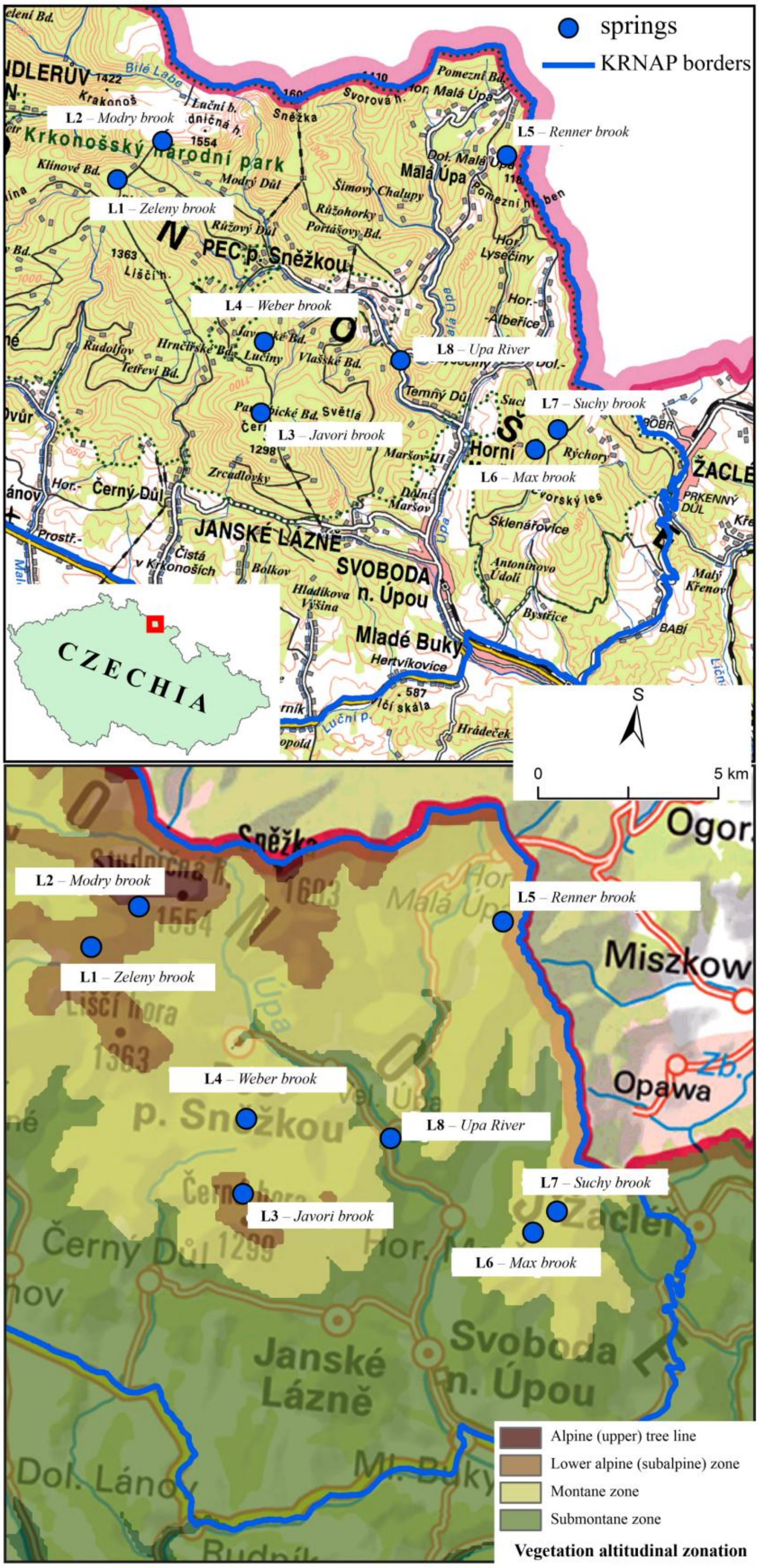

Figure 1. *Distribution map of studied high-mountain springs vegetation in The Krkonoše Mountains National Park (KRNAP – KPN), Czech Republic*

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Data analysis and statistical methods

The springs type was determined by analysing its groundwater environment. Simpson's dominance index and Shannon's diversity index was calculated for each individual location, using the PAST software (Hammer et al. 2001). A TWINSPAN analysis (Hill and Smilauer, 2005) was used to perform the preliminary classification of communities (cluster analysis). Principal component analysis (PCA) of species composition (relevé diversity is expressed as Shannon-Wiener index) with forward selection of environmental factors was analysed by CANOCO 5 software package (Smilauer and Leps, 2014).

Results and discussion

On selected springs, a total of 59 vascular plants and 9 mosses were determined, including 8 endangered taxa among them (*Table 2, Appendix I*). The highest occurrence of endangered species is represented by the springs L2, characterized by the continuous plant cover of *Cardamine amara* subsp. *opicii* (*Fig. 4*) with almost equal coverage of herbaceous and mosses layer. It is a newly discovered locality with this taxon, which is extremely rare and critically endangered. This subspecies is extended, among other things, in the highest mountains of the Western Carpathians (Marhold, 1995). It is a natural habitat without the necessary regular management; question is the occurrence of forest animals. There is also *Montia fontana*, which, according Grabherr and Mucina (1993), due to the overall eutrophication of the landscape and increased productivity of stands from the whole of Europe is receding.

Table 2. *Summary of threatened taxa determined at different springs*

	Statutes by Red List (Grulich 2012)	**Springs**	**Cover-abundance scale**
Cardamine amara subsp. *opicii*	C1 b - Critically threatened taxa	L2	3
Delphinium elatum	C2 r - Endangered taxa	L6	2
Epilobium alsinifolium	C3 - Vulnerable taxa	L2, L4, L8	2, +, 1
Epilobium palustre	C4 a - Lower risk – near threatened	L5	1
Chrysosplenium oppositifolium	C4 a - Lower risk – near threatened	L8	1
Montia fontana subsp. *fontana*	C1 b - Critically threatened taxa	L2	1
Swertia perennis	C2 r - Endangered taxa	L2	+
Viola biflora	C4 a - Lower risk – near threatened	L2, L3, L5	+, r, r

Cover-abundance 7-degree scale follows Braun-Blanquet | L1 – L8 see Materials and methods

Species richness

Fig. 2 (left side) shows highest species richness on L5 and L3 (with 19 vascular plants and 4 mosses, respectively 18 and 3). It is probably connected with this that the index is heavily dependent on the most numerous species and less sensitive to rare species. On the other hand, the lowest diversity was observed on L7 and L4 (5 vascular plants and 2 mosses) – which are closely similar. On right side of *Fig. 2* is a clear cluster with L1 and L2 (subalpine altitude) that also shows in other clusters, as well as springs. First cluster covers L6-L8, localities with lowest altitude (submontane zone).

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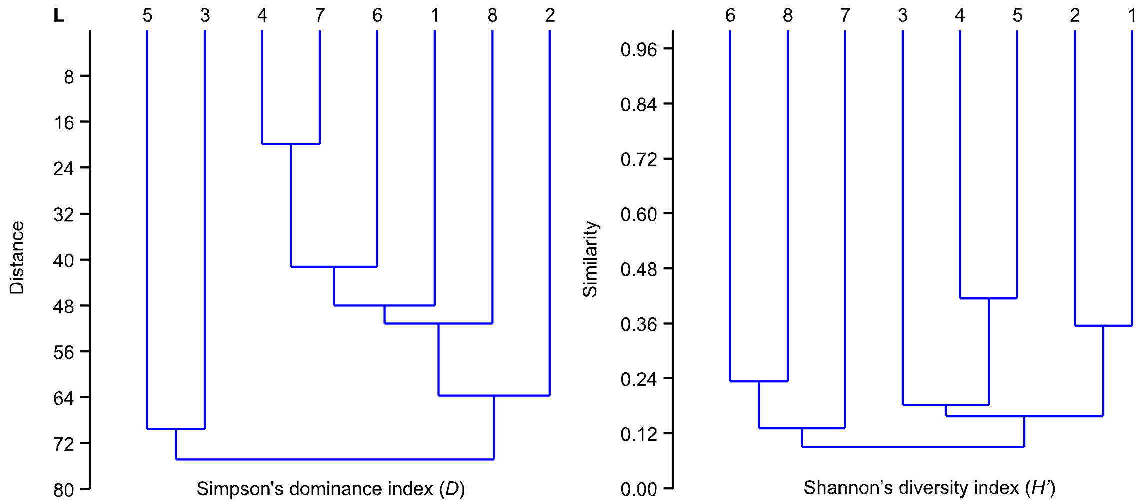

Figure 2. *Cluster analysis of species richness of different springs indicated by Simpson´s dominance index (D) and by Shannon's diversity index (H') - complete linkage (Euclidean distance) | L1 – L8 see Materials and methods*

The results of species diversity and phytocenological relevés show diverse types of communities in the springs. These are communities of the alliance *Swertio perennis-Dichodontion palustris* Hadač 1983 (L1-L4) and associations *Caricetum remotae* Kästner 1941 (L5, L6), *Cardamino-Chrysosplenietum alternifolii* Maas 1959 (L7) and *Pellio epiphyllae-Chrysosplenietum oppositifolii* Maas 1959 (L8).

According to Hajkova et al. (2006) the major species turnover in springs follows the variation in water pH and mineral content in water but altitude remains an important factor in all cases. Our results indicated that springs preserved in managed forests constitute biodiversity hotspots in the landscape because they provide habitat for many species of plants, including protected and endangered (Spalek and Prockow, 2011).

The first and second axis in PCA are explained in 24.62 and 23.72, respectively. *Fig. 3* shows both species abundance and richness by various springs in relation to their environmental gradient. First axis represents the effects of elevation and slope while the second illustrates aspect. The resulting species richness is a function of the combination of altitude and exposure, where the highest species richness values were recorded in the highest located subalpine headwater areas with southern exposures.

This contradicts the conclusions of some studies (Hajkova et al., 2006); that the plant species richness decreases significantly with increasing altitude. On the contrary, Krkonoše Mountains are specific due to rarity of calcareous bedrock. In such conditions, vegetation of the high-mountain springs (*Montio-Cardaminetea* class) is not diversified according to the gradient of mineral richness. Species composition is more influenced by the variation in altitude and also by succession advancing due to *Sphagnum* sp. expansion and peat accumulation (Hajek et al., 2005). As a general conclusion, our results show that subalpine springs vegetation should be analysed separately while searching for vegetation-environment relation.

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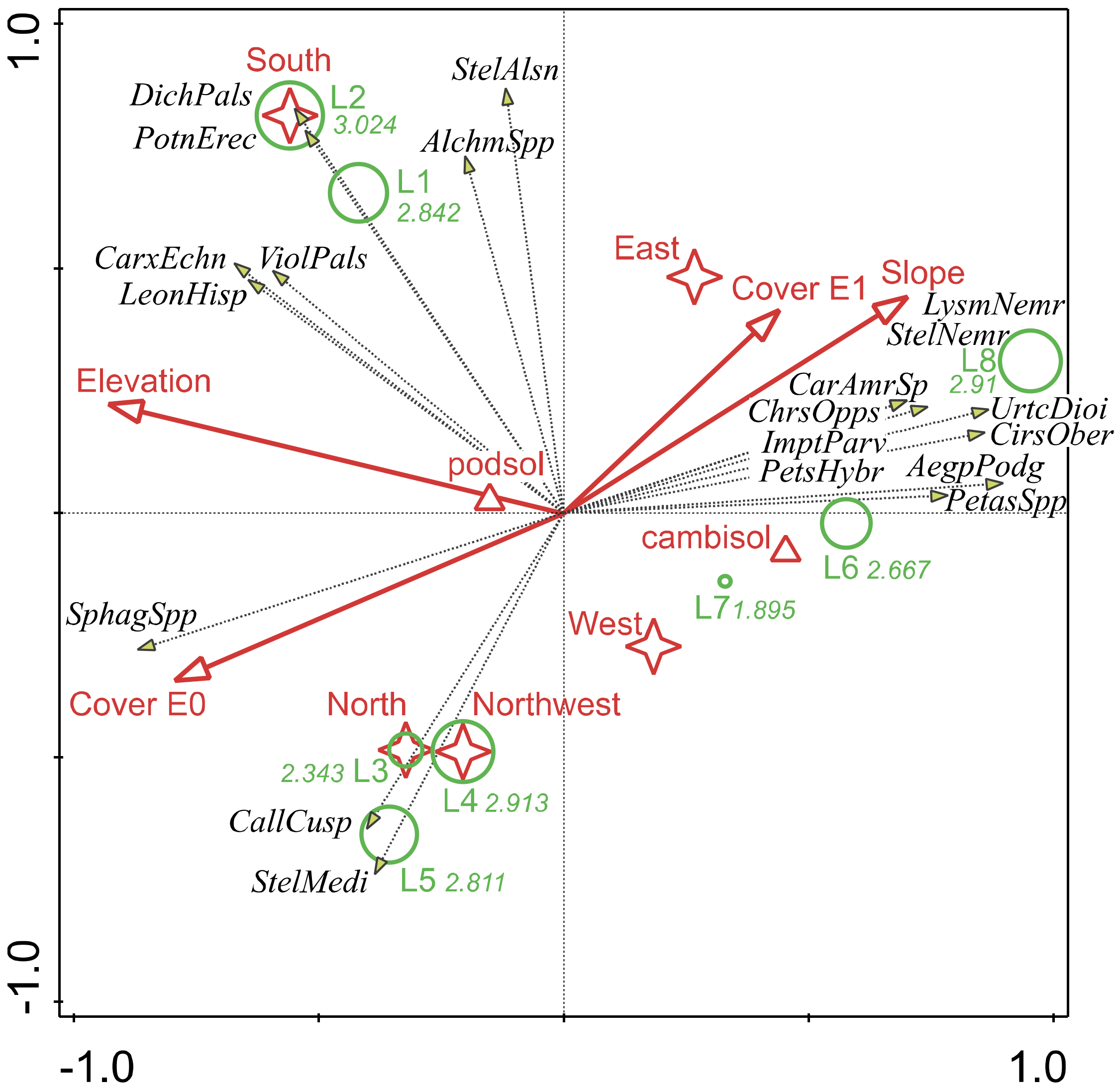

Figure 3. *An ordination diagram of PCA with environmental supplementary variables | red – supplementary variables; black - species; green – springs (L1 - L8) – point size and number correspond with Shannon-Wiener index | L1 – L8 see Materials and methods | AegpPodg - Aegopodium podagarium | AlchmSpp - Alchemilla spp. | CallCusp – Calliergonella cuspidata | CarAmrSp - Cardamine amara | CarxEchn - Carex echinata | ChrsOpps - Chrysosplenium oppositifolium | CirsOber - Cirsium oleraceum | DichPals - Dichodontium palustre | ImptParv - Impatiens parviflora | LeonHisp - Leontodon hispidus | PetasSpp - Petasites spp. | PetsHybr - Petasites hybridus | PotnErec - Potentila erecta | SphagSpp - Sphagnum spp. | StelAlsn - Stelaria alsine | StelMedi - Stelaria media | StelNemr - Stellaria nemorum | UrctDioi - Urtica dioica | ViolPals - Viola palustris | LysmNemr - Lysimachia nemorum*

Management of springs

In terms of the overall approach to management of habitats, springs are distinguished by two basic groups. The first involves the natural habitat (forest and subalpine springs as L1, L2 and L3; bogs and some types of transitional bogs), which should generally leave the spontaneous development with possible one-off interventions aimed primarily at restoring the natural water regime. This corresponds with Barquin and Scarsbrook (2008). The second group consists of semi-natural habitats – mainly springs L4 and L6.

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The existence of these semi-natural habitats is conditioned especially by human activities, including deforestation and subsequent traditional management in the past of non-forest springs, calcareous and non-calcareous moss springs and some types of transitional bogs. These habitats require more or less steady, albeit extensive management to replace the former traditional agricultural practices.

Conclusions

The springs are unique ecotones that integrate ecological characteristics and human impacts associated with both underground and surface water and terrestrial ecosystems. Spring vegetation occurs across a broad altitudinal range from lowlands to the subalpine belt, both in open places and under forest canopies. A clear definition of the management of springs, with the help of vegetation and environmental factors surveys, is a precursor to their effective protection and restoration. Our results suggest that subalpine springs vegetation should be analysed separately with respect to vegetation-environment correlations. Separate analysis of springs below and above timberline is quite appropriate.

Figure 4. *The critically threatened plant Cardamine amara subsp. opicii (large bitter-cress) on subalpine springs Modry brook (L2 | 18. 9. 2015) with protection (fleece) against grazing forest animals*

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APPENDIX

Appendix I. *Shortened synoptic table with Braun-Blanquet scale values*

	Taxon	L1-8		Taxon	L1-8
E_1	*Aegopodium podagaria*	. . . . . 1 + +	E_1	*Montia fontana*	. 1 . . . . . .
	Agrostis canina	. . . + ...		*Myosotis nemorosa*	. . . 1 1 . . .
	Agrostis capillaris	3		*Myostis palustris*	. + ... r
	Agrostis stolonifera	. . . 1 + 1 . r		*Nardus stricta*	2 . . . .
	Anthriscus sylvestris	. . . . . . 1 .		*Oxalis acetosella*	. . . . 2 . 1 +
	Alchemilla spp.	+ + . . . + . .		*Petasites hybridus*	 2
	Athyrium filix femina	. . . . r . . +		*Petasites* spp.	. . . . . 3 + +
	Cardamine amara spp.	. . . . . . r 3		*Poa trivialis*	. . 2 + . + . .
	Cardamine amara subsp. *opicii*	. 3		*Potentila erecta*	1 1 . . . . . .
	Carex echinata	+ 2 . . + . . .		*Ranunculus acris*	. + . + . + . .
	Carex rostrata	. . . . 1 . . .		*Rumex acetosa*	. + . + r . . .
	Cirsium oleraceum	. . . . . + . +		*Rumex alpinus*	1 . . . .
	Crepis paludosa	+ 1 . 1		*Sanguisorba officinalis*	. . . . r . . .
	Dactylorhiza fuchsii	. + ...		*Senecio* spp.	. . . 1 r . . r
	Delphinium elatum	. . . . . 2 . .		*Stellaria alsine*	2 1 . 2 4 . . 1
	Deschampsia cespitoza	. 1		*Stellaria nemorum*	 1
	Dryopteris dilatata	. . + ...		*Stellaria media*	. . + ...
	Dryopteris filix- mas	. . 1		*Swertia perennis*	. + ...
	Epilobium alsinifolium	. 2 . + . . . 2		*Symphytum officinale*	. . . . +
	Epilobium angustifolium	. . . 2 . 1 . .		*Thelypteris limbosperma*	. . . . r . . .
	Epilobium palustre	. . . . 1 . . .		*Urtica dioica*	. . . . . r . +
	Equisetum sylvaticum	. . . + r . . .		*Valeriana dioica*	. . . r . . . +
	Eriophorum vaginatum	2		*Vicia cracca*	. . . . . + . .
	Galium palustre	. . . r r . . .		*Viola biflora*	. + r . r . . .
	Geranium palustre	. . . . . 1 . .		*Viola palustris*	1 + . . + . . .
	Homogyne alpina	1 r 1	E_0	*Calliergonella cuspidata*	+ . + 1 1 . 1 .
	Hypericum maculatum	+ . . . . 1 . .		*Dichodontium palustre*	1 3 . . . . . .
	Chaerophilum hirsutum	2 1 . . 1 3 . 1		*Isothecium alopecuroides*	. . + . . 1 . +
	Chrysosplenium alternifolii	r		*Pellia epiphyla*	. . 1 . . . . .
	Chrysosplenium oppositifolium	 1		*Philonotis fontana*	. 2 . . . . . .
	Impatiens parviflora	 2		*Polytrichum commune*	. 1 2 1 1 + . .
	Juncus effusus	. . . 1 + . . .		*Polytrichum formosum*	1 . . . .
	Leontodon hispidus	+ + . + ...		*Rhizomnium punctatum*	. . 2 . . . + .
	Lysimachia nemorum	 1		*Sphagnum* spp.	2 2 4 2 3 . . .

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THE EFFECT OF DIFFERENT IRRIGATION REGIMES ON FLORET AND ROOT ANATOMY OF AEROBIC RICE GENOTYPES IN KHUZESTAN, IRAN

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Abstract. The aim of this study was to examine the effect of different planting regimes on anatomical traits of floret and root effective on productivity and seed hollowness as well as grain yield of 12 aerobic rice genotypes in subplots under the influence of four different irrigation regimes (irrigation intervals of 1, 3, 5 and 7 days) in the main plots by split-plot based on randomized complete block with three replications for two years (2014 and 2015) in Khuzestan. Results of combined analysis showed that there were significant differences in all parameters between genotypes, irrigation regimes and the interaction of the two factors except for the stigma length and the central cylindrical area of roots. Reduction of the amount of irrigation had decreased central cylindrical area and xylem of root in order to increase efficiency and maintain the water potential in plants. The highest length of anthers and stigmas was observed in the 5-day irrigation regime and maximum width of anthers, style length and pollen area was observed in the 3-day irrigation regime and the genotypes had the highest grain yield (5094.31 kg/ha) due to the ideal conditions. Therefore, it can be concluded that by focusing revision goals on reducing the central cylinder and xylem areas in order to increase the efficiency of irrigation water in terms of reduced irrigation and controlling lengths of florets parts we can hope to increase connection between pollen and flower and finally fertility to increase production under different irrigations.
Keywords: *rice, irrigation regimes, anatomy, cluster, floret*

Introduction

Rice is one of the oldest cultivation plants (Ghosh and Chakma, 2015) and is the main food of more than half the world's population (Park et al., 2014). Drought is a serious risk for the successful production of major crops in the world, especially rice, which can occur at any time during the growing season. Hence, one of the main challenges in agriculture Is to produce more food with less water (Tuyen and Prasad, 2008). Seventy percent of world's fresh water is consumed in agriculture, which 25 to 30 percent of this amount is devoted to rice cultivation (Sedaghat et al., 2015). Rice has the greatest amount of water consumption among agricultural products and about 80% of Asia's fresh water is used for this (Sedaghat et al., 2015). Almost 75% of world's rice is obtained from irrigated rice paddies (Carmelita et al., 2011). Irrigation with few days interval provides enough oxygen for the plant root system, which this would accelerate the mineralization of organic matter and soil nitrogen-fixing. All of these improve

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increasing in the plant nutrients and thus increase its growth (Dong et al., 2012). Lack of moisture is one of the most important growth limiting factors (Mosavy et al., 2016). Intermittent irrigation management can provide plant needs in crisis situations (Shanmugasundaram, 2015). The main advantage of intermittent irrigation with intervals of few days is savings in water consumption (Uphoff et al., 2013). Limuchi et al. (2014) reported the reduction of growth period length in order to decrease the un-structured carbohydrates to the main tank, i.e. grain, as an important factor in reduced yield (Limuchi et al., 2014).

Anatomical changes in plants under environmental stress can reduce the vascular bundles area such as Xylem area, which the result of these changes can protect cells from death and dieback (Mostajeran et al., 2008).

There was a difference in terms of pollen germination at times after anthers' opening. Variety Minyhui 63 had the highest germination rate (85%) at time 0 or immediately after anthers' opening; But variety Rufipogon with lower germination rate (60%), 50% of pollens were alive after 12 min (longer). The lowest germination rate (34%) was observed in hybrid varieties with an average longevity that more than 50% were alive after 10 min even after 40 min (Song et al., 2001). Uniformity between opening of florets and anthers causes high self-pollination in rice, but stressful situations at the time of flowering can postpone the turgidity and growth of pollens (Matsui et al., 2000). Matsui and Hisashi (2003) showed that there were positive correlation between the number of pollen on stigma and morphological characteristics.

Many researches were carried out on the root structure and its effect on yield in drought conditions and the structural changes and root anatomy were studied in irrigation conditions and the best cross-section due to irrigation conditions for example, increasing and decreasing cross-section in order to increase water absorption and increase efficiency in drought conditions and selection should be done from genotypes with small cross-section in order to increase water use efficiency in root (Lopes and Reynolds, 2010; Hamadanzadah et al., 2015). It should be stated the management practices of different irrigation should be overcome to increase the volume and finally cross-section of root (Sadranasab et al., 2014). In terms of root characteristics it can be stated that the desirable root characteristics change with different irrigation conditions and modification to increase cross-section, central cylinder and xylem of root to absorb more water in favorable irrigation conditions (Zhou et al., 2007; Moshafaghi et al., 2014). The root is the only organ in direct contact with the surrounding soil, so having an efficient xylem bundles and central cylinder appropriate to the circumstances and anatomical changes in order to organs adaption according to previous statements can have an effective role in dealing with decreased water or watering more than plants need in line with the absorption of nutrients and minerals and finally can fill the main plant reservoir, i.e. grains (Sadat Jamali et al., 2014; Shelden et al., 2013). To increase their survival under conditions of drought and temperatures, plants change anatomically such as reduced vascular bundles area for better compatibility with the environment (Ghorbani et al., 2011).

Increased drought to maintain the water potential in plant causes a reduction in diameter of Xylem vascular bundles (Majd et al., 2009). Xylem vascular bundles area decreases in different conditions leading to water stress in order to improve irrigation efficiency and to maintain plant water potential (Limouchi and Farahvash, 2014). Stresses can decrease vascular bundles area and diameter to improve water use efficiency and to reduce transpiration, while these traits are positively and significantly

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correlated to grain yield (Limouchi, et al., 2013). Water shortage for rice leads to a reduction in the value of anatomical traits effective in water retention and movement such as vascular bundles to maintain the amount of water in rice (Daxylem, 2016).

This study aimed to identify the tolerance and sensitivity mechanisms to drought in different irrigation intervals in order to increase florets fertility and reduce hollowness as well as to study changes in anatomical characteristics of roots.

Materials and methods

This study aimed to reduce and optimize water consumption and increase irrigation efficiency in split-plots by two factors and 3 replications in a randomized complete block design by upland rice method (3 × 4 m plots) for two years (2014 and 2015) in Shavoor Agricultural Research Station of Agriculture and Natural Resources Research Center of Iran in Khuzestan province at 70 km north of Ahvaz between the rivers of Karkhe and Karun (31° 50′ latitude and 48° 28′ longitude, 33 m above sea level). Farm soil texture was loamy-clay with pH = 7-7.5 and electrical conductivity of 2.5 mmhos/cm, and amounts of nitrogen, phosphorus, potassium and zinc were 0.09%, 10-12, 120 and 2.5 ppm, respectively. Four irrigation regimes include intermittences of one-day or control (common in the region) (I1), 3 (I2), five (I3) and seven days (I4) as main factor levels and 12 rice genotypes were places in subplots (*Table 1*).

Table 1. *Some features and pedigree of the genotypes used in the study*

Genotype	Cross	Origin	Drought Tolerance
VANDANA	C 22/KALAKERI	INDIA	1
IR 78908-193-B-3-B	VANDANA/IR 65	IRRI	1
IR 81429-B-31	IR 78908-44/IR 78908-86	IRRI	1
IR 78875-176-B-1-B	PSB RC 9/IR 64	IRRI	3
IR 79971-B-202-2-4	VANDANA/WAYRAREM	IRRI	5
IR 80508-B-194-4-B	PSB RC 9/AUS 257	IRRI	7
IR 80508-B-194-3-B	PSB RC 9/AUS 257	IRRI	5
IR 79907-B-493-3-1	IR 55419-04/IR 64	IRRI	5
IR 81025-B-347-3	NSIC RC 140/IR 74371-3-1-1	IRRI	5
IR 81025-B-327-3	NSIC RC 140/IR 74371-3-1-1	IRRI	3
NADA	$AMOL_3$ / SANG TARAM	IRAN	3
TAROM	–	IRAN	9

The numbers from 1 to 9 have the highest and lowest drought tolerance, respectively.

After soil preparation, dry seeds of each genotype were sown in 20 cm rows by a Hamadani farmer and then irrigation regimes were applied since the mid-tillering. Plots were irrigated to a height of 5 cm with water supplied by a pump and then irrigation stopped. This trend was applied for whole growing season and all four treatments. To prevent water infiltration into adjacent plots, all stacks to a depth of 1 m into the soil and irrigation streams' walls were covered by a plastic. Irrigation regimes were selected according to the conditions and potential of water and the amount of water entering the plots was determined according to the water height and plots' size during irrigation, which was about 7 h, as well as regarding the water discharge. Some meteorological

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parameters are shown in *Table 2*. To provide nutrients; nitrogen of urea source was used by 200 and 300 kg/ha as 25% baseline (20-25 days after emergence) and the remaining 75% in three times (25%) as first to third first input respectively in the first bud cluster formation (35 to 40 days after using baseline fertilizer), The seed formation (30-35 days after the first first input) and 50% in time of the emergence of clusters 50 kg of phosphorus fertilizer per ha from triple superphosphate source, 100 kg of potash fertilizer and 40 kg zinc per ha from sulfate source were used in the soil. Integrated weed control including weeding and using 2,4-D pesticide (1.5-2 L/ha; 35 to 40 days after emergence) was applied. The following traits were studied.

Table 2. *The minimum and maximum average of monthly temperature (sowing to harvesting) in Shavoor Agricultural Research Station for two years (2014 and 2015)*

Month	(2015)		(2014)	
	Mean min. (°C)	**Mean min. (°C)**	**Mean min. (°C)**	**Mean min. (°C)**
Jun.	26	44	26.6	46.2
Jul.	27.8	46.7	27.8	45.7
Aug.	27.8	46.5	29.1	47.5
Sep.	25.2	44.5	27.4	44.6
Oct.	21	38	22.2	39.5
Nov.	12.7	29	15.8	27.8
Average	23.42	41.45	24.82	41.88

Matsui and Omasa method (Matsui and Omasa, 2002) was used to determine the length and width of anthers as well as length of stigma and style. One day before the opening of florets, 1/3 of florets from the tip of first cluster branch (18 anthers from three florets) were collected for each variety. Measurements were done under a microscope after stabilizing in F.A.A mix (Formalin-acetic acid-alcohol). Anther length from longer side of anther sac and anther width from average of two sides, one parallel to anther wall that opens after maturity (W1) and the other perpendicular to it (W2), were taken. Measuring length of stigma and style and counting pollen on the stigma were performed after staining 15 florets with Cotton blue for 15 min. Then stained samples were placed on a lamel to prepare images and traits were measured with a light microscope with a magnification of 40. Length of stigma and style were measured from the tip of stigma to the junction with style and from base of stigma to the junction with ovary, respectively. Viability, diameter and area of pollens were determined by using the Erie classification system based on their shape and staining with potassium iodide (Anonymous, 1982).

For the measurement of anatomical traits, roots were taken out of the soil at the time heading and then 2-3 cm slices in middle parts were prepared. F.A.A solution was used to store samples. Cross- section and thin slices were manually prepared using Styrofoam and to stain cross- sections of roots in order to observe and measure the central cylindrical area and xylem, samples were washed with distilled water and placed in bleach for 15 min and then in Carmen Zaji for 20 min and finally in green methyl for 10-15 s and they were washed thoroughly with distilled water between each stage. Then stained samples were placed on a lamel to prepare images and traits were measured with a light microscope with a magnification of 40 (Limouchi, 2013).

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Measurement of the grain yield at 14% moisture (the moisture after harvest) was done after reaching 85% grain in harvest cluster in an area of 1.5 m^2 in the middle of each plot and removing borders.

Dry matter per ha (after placement of samples without base for 72 h in an oven), fertility rate (the ratio of filled florets to total florets) and the weight of one thousand seeds by randomly picking 30 main clusters from each replication were measured.

All data obtained from the study after ensuring normality were analyzed using analysis of variance (combined) and correlation in SAS and SPSS and Duncan test was used for mean comparison of data at 5% level.

Results and discussion

Anther length

In this study, it was found that there were significant differences between years, irrigation regimes and genotypes and the interaction of irrigation regimes and genotypes at 1% level and also between the interaction of years and genotypes and interaction of years, irrigation regimes and genotypes at 5% level; but other factors were not significantly different (*Table 3*).

Table 3. *Combined analysis results of floret anatomical characteristics in study treatments*

		MS				
S.O.V	**df**	**Anther width**	**Anther length**	**Stigma length**	**Style length**	**Pollen area**
Year	1	401120.641**	41744.216**	558912.021**	52604.333**	189216.370**
Rep. (year)(error a)	4	258802.043	17987.262	239490.315	18758.126	153330.443
Irrigation regimes	3	59824.894**	26521.502**	8291.879*	4704.412**	180214.624**
Irrigation regimes × year	3	1421.210^{ns}	130.924^{ns}	2091.471^{ns}	2787.541**	14708.122^{ns}
Error b	12	742.543	64.848	922.474	2060.920	20744.415
Genotypes	11	640247.613**	189749.713**	3644030.377**	939295.339**	1912811.473**
Genotypes × year	11	9226.510*	573.303**	9172.964**	1244.822**	7975.587^{ns}
Irrigation regimes × genotypes	33	9611.064**	3315.760**	1182.420^{ns}	4301.277**	11582.615*
Irrigation regimes × year × genotypes	33	6804.544*	307.815**	3518.685*	658.234**	11618.506*
Error c	176	3984.748	161.880	2198.123	366.246	6796.868
C.V (%)	-	5.407	4.734	4.777	5.305	8.830

^{ns}, * and **: Nonsignificant, and significant at 5 and 1% level of probability, respectively

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According to mean comparison, the highest and lowest anther lengths were related to the third (5-day interval) and the first (1-day interval) irrigation regimes, respectively (*Table 4*). The interaction between the two factors is shown in *Figure 1* (*a* and *b*), which genotypes reaction was entirely dependent on irrigation regimes so that genotype IR 78908-193-B-3-B with high tolerance to drought had the longest anther in all irrigation regimes due to the genetic reasons, and the lowest length (1448.271 µm) was seen in the second irrigation regime. In other irrigation regimes, anther length of this genotype was in one statistic class. The lowest length of anther (930.056 µm) was seen in Tarom in the first irrigation regime (1-day interval), which had the least drought tolerance among the studied genotypes. It seems that based on genotypes resistance to drought, reduced anther length with increased contact with pollen and prevention of wasting pollen fertility could increase fertility and grain yield. Thus, increasing performance of genotype IR 78908-193-B-3-B in 3- day interval irrigation and Tarom in 1- day interval irrigation can be due to the reduced length of anthers, increased fertility efficiency and trapping pollens (*Table 5*).

Table 4. *Mean comparison of floret anatomical characteristics*

Treatments	Anther length (µm)	Anther width (µm)	Stigma length (µm)	Style length (µm)	Pollen area (µm^2)
Irrigation regimes					
I1	1134.149 d	277.672 a	970.172 b	364.426 a	955.499 ab
I2	1157.250 c	279.879 a	974.414 b	370.238 a	986.675 a
I3	1202.256 a	277.406 a	989.771 a	355.507 a	922.476 b
I4	1175.717 b	239.998 b	991.428 a	352.687 b	869.657 c
Genotypes					
VANDANA	1110.980 f	318.097 c	567.976 g	490.643 c	1137.886 b
IR 78908-193-B-3-B	1481.819 a	368.137 b	1360.728 b	837.301 a	765.051 e
IR 81429-B-31	1373.085 b	230.297 e	584.722 fg	239.530 h	1255.353 a
IR 78875-176-B-1-B	1123.656 f	168.841 i	1668.796 a	641.445 b	661.674 f
IR 79971-B-202-2-4	1224.765 d	198.580 g	791.294 e	240.386 h	584.730 g
IR 80508-B-194-4-B	1172.364 e	312.091 c	782.144 e	315.958 e	1261.110 a
IR 80508-B-194-3-B	979.791 i	465.234 a	1184.453 d	173.422 i	1226.431 a
IR 79907-B-493-3-1	1229.204 d	314.409 c	1287.965 c	267.249 f	1001.949 c
IR 81025-B-347-3	1062.733 g	253.969 d	1284.054 c	255.775 fg	1024.589 c
IR 81025-B-327-3	1020.734 h	208.821 f	1173.084 d	260.136 fg	985.301 c
NADA	1297.232 c	208.613 f	605.581 f	353.342 d	911.373 d
TAROM	931.753 j	177.778 h	486.559 h	253.385 g	387.473 h

Means in each column, followed by at least one similar letter(s) are not significantly different at 5% probability level using Duncan’s Multiple Range Test
I1, I2, I3 and I4 include irrigation regimes of 1, 3, 5 and 7 days, respectively

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Figure 1. *Floret anatomy; a: pollen received by the stigma; b: pollen fertility*

Table 5. *Mean comparison of floret anatomical characteristics in study treatments*

Treatments: Irrigation regimes	Treatments: Genotypes	Anther length (µm)	Anther width (µm)	Stigma length (µm)	Style length (µm)	Pollen area (µm^2)
I1	VANDANA	1071.510 h-k	291.176 ij	559.159 gh	491.345 f	1184.520 a-d
	IR 78908-193-B-3-B	1489.175 a	390.067 e	1360.874 b	853.066 ab	745.707 l-p
	IR 81429-B-31	1364.309 a-d	220.771 m-p	580.830 gh	232.526 p-t	1250.097 ab
	IR 78875-176-B-1-B	1057.614 h-k	160.507 s-u	1662.246 a	619.957 e	663.791 o-q
	IR 79971-B-202-2-4	1179.800 f-h	213.384 m-q	791.225 f	229.144 p-t	582.333 qr
	IR 80508-B-194-4-B	1143.127 f-i	332.787 f-h	756.276 f	343.755 k-j	1311.327 a
	IR 80508-B-194-3-B	956.229 kl	493.116 a	1158.657 e	201.201 tu	1280.356 ab
	IR 79907-B-493-3-1	1174.112 f-h	334.953 f-h	1277.590 b-e	289.868 k-m	1051.923 d-h
	IR 81025-B-347-3	1011.152 i-l	290.735 ij	1251.706 b-e	271.977 k-p	1100.482 c-g
	IR 81025-B-327-3	999.617 j-l	199.265 n-r	1172.208 c-e	251.145 m-s	997.017 f-i
	NADA	1260.090 c-f	209.217 n-r	603.849 g	352.276 g-i	878.155 i-l
	TAROM	903.056 l	196.089 o-r	467.445 h	236.851 o-t	420.279 st
I2	VANDANA	1066.018 h-k	340.933 fg	552.512 gh	484.741 f	1235.021 a-c
	IR 78908-193-B-3-B	1448.271 ab	397.428 de	1354.867 b	812.564 c	819.228 k-n
	IR 81429-B-31	1341.588 b-d	229.725 l-o	589.862 gh	213.975 r-u	1287.564 ab
	IR 78875-176-B-1-B	1062.295 h-k	175.526 r-u	1620.856 a	626.857 e	689.605 n-q
	IR 79971-B-202-2-4	1190.020 e-h	210.576 m-r	775.121 f	236.709 o-t	758.382 l-p
	IR 80508-B-194-4-B	1159.017 f-h	314.128 g-i	770.769 f	311.118 i-k	1287.022 ab
	IR 80508-B-194-3-B	981.252 j-l	483.203 ab	1183.394 c-e	185.586 uv	1251.348 ab
	IR 79907-B-493-3-1	1285.357 c-f	313.941 g-i	1278.831 b-e	273.749 k-p	1013.444 f-i
	IR 81025-B-347-3	1078.018 h-k	258.708 j-l	1291.534 b-d	279.273 k-o	1056.018 d-h

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	IR 81025-B-327-3	995.436 j-l	232.734 l-n	1191.761 c-e	288.035 k-n	1051.923 d-h
	NADA	1323.895 b-e	209.957 m-r	593.706 gh	390.169 g	987.433 f-i
	TAROM	955.839 kl	191.689 p-s	489.759 gh	340.073 h-j	403.112 st
I3	VANDANA	1156.019 f-h	351.963 f	578.583 gh	496.488 f	1027.005 e-h
	IR 78908-193-B-3-B	1496.416 a	426.277 cd	1365.090 b	815.574 bc	792.220 l-o
	IR 81429-B-31	1396.225 a-c	256.923 kl	585.102 gh	258.930 m-r	1277.021 ab
	IR 78875-176-B-1-B	1183.192 f-h	186.852 p-s	1694.725 a	640.921 de	682.299 n-q
	IR 79971-B-202-2-4	1258.220 c-f	189.865 p-s	798.640 f	251.689 m-s	511.105 rs
	IR 80508-B-194-4-B	1200.053 e-h	304.092 hi	794.855 f	303.911 j-l	1243.091 a-c
	IR 80508-B-194-3-B	992.817 j-l	455.074 bc	1193.440 c-e	156.494 v	1215.020 a-c
	IR 79907-B-493-3-1	1280.022 c-f	309.848 g-i	1299.556 bc	265.727 l-q	989.275 f-i
	IR 81025-B-347-3	1151.320 f-h	244.733 lm	1294.880 bc	236.851 o-t	975.856 f-j
	IR 81025-B-327-3	985.865 j-l	211.761 m-q	1165.186 de	256.810 m-s	1013.203 f-i
	NADA	1363.598 a-d	213.674 m-q	609.925 g	357.070 gh	942.260 h-k
	TAROM	963.322 j-l	177.811 q-u	497.267 gh	225.617 q-u	401.352 st
I4	VANDANA	1150.373 f-h	288.314 i-k	581.651 gh	490.000 f	1105.000 c-f
	IR 78908-193-B-3-B	1493.415 a	258.775 j-l	1362.081 b	868.000 a	703.048 m-q
	IR 81429-B-31	1390.220 a-c	213.768 m-q	583.095 gh	252.687 m-s	1206.728 a-c
	IR 78875-176-B-1-B	1191.522 e-h	152.481 tu	1697.357 a	678.046 d	611.000 p-r
	IR 79971-B-202-2-4	1271.021 c-f	180.494 q-t	800.190 f	244.000 n-t	487.101 rs
	IR 80508-B-194-4-B	1187.260 e-h	297.355 i	806.677 f	305.047 j-l	1203.000 a-c
	IR 80508-B-194-3-B	988.868 j-l	429.543 c	1202.319 c-e	150.409 v	1159.000 b-e
	IR 79907-B-493-3-1	1177.327 f-h	298.896 i	1295.883 bc	239.652 o-t	953.155 g-k
	IR 81025-B-347-3	1010.443 i-l	221.700 m-p	1298.097 bc	235.000 o-t	966.000 f-j
	IR 81025-B-327-3	1102.019 g-j	191.524 p-s	1163.183 de	244.553 n-t	879.060 i-l
	NADA	1241.345 d-g	201.604 n-r	614.842 g	313.854 i-k	837.644 j-m
	TAROM	904.794 l	145.523 u	491.767 gh	211.000 s-u	325.150 t

Means in each column, followed by at least one similar letter(s) are not significantly different at 5% probability level using Duncan's Multiple Range Test
I1, I2, I3 and I4 include irrigation regimes of 1, 3, 5 and 7 days, respectively

Due to the fact that starch degradation and the availability of non-structural carbohydrates are essential for formation of anther wall and elongation together with the generative (producing pollen), it seems that depending on the irrigation regime the relative contribution of these carbohydrates is different in formation of anther parts. The results did not correspond to Suzuki (1981) on larger anthers had more pollens that can compensate the reduction of number of germinated pollen and thereby increase the tolerance to thermal stress, because in addition to pollen number, their size and the water potential of each pollen to create pressure for anther opening and pollen releasing are also factors for increasing fertility.

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Correlation coefficients results showed that length negatively correlated with yield (-0.012), which can be due to the longer length of anther for transferring pollen from the anther to the stigma and this corresponded to other studies (Arvin and Vafabakhsh, 2016; Akbari et al., 2016) on high effect of increased fertility on grain yield (*Table 6*).

Table 6. *Correlation coefficients between grain yield and traits related to floret anatomy of rice genotypes*

	1	2	3	4	5	6
1-grain yield	1	-0.012	0.027	0.139*	-0.067	0.038
2-anther length		1	0.141*	0.095	0.462**	0.129*
3-anther width			1	0.224**	0.093	0.545**
4-stigma length				1	0.404**	-0.019
5-Style length					1	-0.204**
6-pollen area						1

* and **: Significant at 5% and 1% probability levels, respectively

Anther width

Results of combined analysis showed that differences between factors except for interaction between year and irrigation regimes (*Table 3*). According to mean comparison, anther width was in one level for three irrigation regimes (1-, 3- and 5-day interval) shown in *Figure 1* (*a* and *b*), but significant decrease in anthers width was seen in the fourth irrigation regime (7 day interval) due to the reduced nutrients transportation and lack of growth and this could be one of the reasons of grain yield reduction due to decreased diameter suitable for the pollen passage. Genotype IR 80508-B-194-3-B had the widest anther in all irrigation regimes, which the highest width (493.116 μm) was seen in the first irrigation regime (1-day interval) and the lowest width was seen in the fourth irrigation regime (7-day interval) and the lowest width in this irrigation regime belonged to Tarom (145.523 μm) (*Fig. 1, Tables 4* and *5*). Comparison of changes trends in fertility and anther width showed it was a slightly different trend or in other words, having the widest anther does not lead to higher fertility. Although it seems that wider and longer anthers have more potential for more pollens. The above results corresponded to those of Mamnan et al. (2009).

Stigma length

In this study it was found that the effect of years, genotypes and the interaction of years and genotypes were significant at 1% level and the effect of irrigation regimes and the interaction of years, irrigation regimes and genotypes were significant at 5% level but no significant difference was observed for other effects (*Table 3*). Mean comparisons showed that increasing irrigation interval had increased stigma length, so that the longest stigma (991.428 μm) was observed in the fourth irrigation regime (7-day interval) (*Table 4*). Due to the interaction of two factors, the maximum length of stigma was related to genotype IR 78875-176-B-1-B, which was in one statistical level in all irrigation regimes. The minimum length of stigma was related to Tarom, which reduction in irrigation intervals affected stigma length. The results indicated the

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presence of genetic differentiation between them and it can be concluded this difference was due to the plant internal and genetic factors that requires more attention to breeding and improvement of rice genotypes in order to optimize the length of stigma with anther length to enhance pollen contact and fertility and finally raising grain yield (*Table 5*). Comparing length of anther and stigma the fourth irrigation regime (7-day interval) suggested an almost same trend and other reasons were reduced nutrients absorption and as a result reduced anther growth. Irrigation regimes with longed anthers had longer stigmas. But the process was quite different among the genotypes and genotypes with longer anthers had shorter stigmas. The results regardless of water conditions can be due to stigmas' morphological differences in receiving and holding pollens. On the other hand, irrigation regimes with larger anthers and longer stigmas had more pollen, which was consistent with Suzuki (1981) on the correlations between the length of stigmas and pollen number. Relative comparison of anthers and stigmas in the fourth irrigation regime suggested a same trend between them and irrigation regimes with longer stigmas had longer anthers. However, this trend was not constant among genotypes and it seems this result is due to the difference between genotypes in terms of stigma morphological characteristics. The above results were consistent with Oyiga et al. (2010) (*Figure 1*).

Stigma length was positively correlated with grain yield (0.139*). It seems that increased length had caused less distance between stigma and anther and hence receiving more pollen, fertility and grain yield, which corresponded to (Oyiga et al., 2010) (*Table 6*).

Style length

The results showed that all factors were significantly different at 1% level (*Table 3*). The second irrigation regime (3-day interval) and the fourth irrigation regime (7-day interval) had the longest and shortest styles, respectively (*Table 4*). The interaction between genotype and irrigation regimes the differences were very diverse and resistance genotypes had longer styles. Unlike genotypes with less resistance, genotype IR 78908-193-B-3-B, which had the longest style in all irrigation regimes, had the longest style (868 μm) in the fourth irrigation regime (7-day interval) and the shortest styles were seen in genotypes with lower resistance. Increasing the planting space affected the style length, so that genotype IR 80508-B-194-3-B had the shortest styles (150.409 μm) in the fourth irrigation regime (7-day interval) (*Table 5*). Given that the time required for pollen tube to reach embryo sac is affected by the irrigation amount, the relative comparison of the length of style and stigma (Takeoka et al., 1992) showed that the style length in the fourth irrigation regime (7-day interval) was slightly higher than other irrigation regimes. The length of pollen tube was very short despite the fact that the lengths of anthers and stigma were high. With improving irrigation conditions in 3-day interval regime at the time of pollination and fertilization, the style length increased. Genotype IR 80508-B-194-3-B with shortest style had a good fertility rate. According to the results, it seems that in addition to anthers' size, the release of pollen and stigmas power to hold pollens, the role of style after the process of pollination is very important for fertilization and seed production. The results corresponded to (Oyiga et al., 2010) on increased length of style in favorable conditions (*Figure 1*).

Correlation coefficients showed this trait had a significant correlation with anthers length (0.462**), stigmas length (0.0404**) and pollen area (-0.204**), which shows the positive and negative effects on these traits because of the increased length of these

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traits together led to perfect adjustment of female organs to get the pollen from the male organ. However, increased pollen area because of inverse relationship with increases style diameter can decline the transferring or accelerating of pollen transport to the ovary, and finally fertility and increased grain yield. Recent comments are consistent with others (Arvin and Vafabakhsh, 2016; Akbari et al., 2016) (*Table 6*).

Pollen area

Results of combined analysis showed that the effect of year, irrigation regimes and genotypes were significant at 1% level and the interaction of irrigation regimes and genotypes, year and irrigation regimes and genotypes were significant at 5% level, but in other cases the differences were not observed (*Table 3*). The mean comparisons revealed that the highest and lowest pollen areas were seen in the second irrigation regime (986.675 μm) and in the fourth irrigation regime (869.657 μm), respectively (*Table 4*). The interaction between the two factors, genotype IR 80508-B-194-4-B with low resistance to drought had the highest pollen area in all irrigation regimes that decreased with increasing irrigation like other genotypes with low resistance, while increased pollen area was seen in genotypes with higher resistance in 3-day irrigation regime, which can be one of the main reasons for increasing grain yield due to having more nutrients energy in larger pollens and more strength to reach the ovary and pollen fertility (*Table 5*). Comparing the size and number of pollens in different planting regimes confirms that the second irrigation regime (3-day interval) had led to larger but less pollens. The pollen fertility can be showed based on the interaction between the amount of starch in pollen and iodide compound, which the pollen staining rate was very high in the second and third irrigation regimes and pollen were mainly globular with dark color. However, the pollen staining rate was low in the fourth irrigation regime (7-day interval) and a lot of damaged pollens with abnormal shape were observed. Recent results consistent with Sing et al. (2005). In addition, these results consistent with Erie classification system (Anonymous, 1982) that showed pollens were globular with dark color.

According to *Table 6,* the pollen area had a positive and significant correlation with male organ in terms of the same influence on all male organs in one direction and also with the style length that had to travel a long distance to reach the ovary as well as the reduction of large pollens entry to the longer styles with reduced diameter are the reasons for lack of entry and thus fertility and grain yield, a significant negative correlation was seen (-0.204**). The results consistent with other studies (Arvin and Vafabakhsh, 2016; Akbari et al., 2016).

Central cylindrical area of roots

In terms of the characteristics associated with root, it can be stated that the desirable characteristics of a root system can change with different soil and climatic conditions (Moshafaghi et al., 2014). In this study it was found that the effect of year, genotypes and irrigation regimes and interaction between year and genotypes were significant at 1% and the simultaneous effect of three year, irrigation regimes and genotypes was significantly different at 5% (*Table 7*). Increasing irrigation intervals resulted from increasing drought had reduced the central cylindrical area due to loss of vascular bundles area inside it due to better water conservation and efficiency (*Table 8*).

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Table 7. *Combined analysis results of root anatomy, yield and yield components in study treatments*

S.O.V	Df	MS					
		Central cylindrical area	**Xylem area**	**Weight of one thousand seeds**	**Fertility rate**	**Dry matter yield**	**Grain yield**
Year	1	532301934.871**	649802.910**	10.975**	450.530*	25853424.092**	13560464.565**
Rep. (year) (error a)	4	217099458.739	370515.839	0.038	522.297	2561996.467	989008.720
Irrigation regimes	3	121782699.834**	782288.739**	16.219**	10234.224**	145357543.359**	39098649.286**
Irrigation regimes × year	3	1895166.349ns	859.876ns	0.309ns	60.241ns	2388814.301*	2585374.714**
Error b	12	702342.089	7930.907	0.683	135.897	1492151.462	753853.818
Genotypes	11	3993166519.985**	2968066.061**	41.780**	6099.922**	43916270.470**	9638043.784**
Genotypes × year	11	13514833.748**	9317.783ns	0.366**	21.869ns	447358.432ns	597461.724ns
Irrigation regimes × genotypes	33	2457257.675ns	60467.631**	1.662**	1028.451**	8971677.302**	3136384.190**
Irrigation regimes × year × genotypes	33	3617810.849*	10606.808ns	0.402**	10.192ns	638523.996ns	199931.307ns
Error c	176	2198614.595	10228.635	0.463	66.664	735416.732	432662.206
C.V (%)	-	4.976	11.700	3.412	12.223	6.726	15.39

ns, * and **: Nonsignificant, and significant at 5 and 1% level of probability, respectively

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Table 8. *Mean comparison of two years related to the anatomy of root, yield and yield components*

Treatments	Central cylindrical area (μm^2)	Xylem area (μm^2)	Weight of one thousand seeds (g)	Fertility rate (%)	Dry matter per hectare (kg/h)	Grain yield (kg/h)
Irrigation regimes						
I1	31077.159 a	985.616 a	19.787 b	78.882 a	12819.270 c	4100.79 c
I2	30642.669 b	909.092 b	20.394 a	73.340 b	14049.324 a	5094.31 a
I3	29185.438 c	814.103 c	20.290 a	63.125 c	13374.369 b	4548.26 b
I4	28265.208 d	748.750 d	19.369 c	51.828 d	10756.589 d	3351.51 d
Genotypes						
VANDANA	23924.493 h	945.349 d	20.644 bc	54.056 e	11177.049 e	3541.29 de
IR 78908-193-B-3-B	18564.500 k	425.653 h	19.783 ef	84.030 ab	13276.174 bc	4303.04 b
IR 81429-B-31	31880.354 d	515.497 g	19.484 fg	80.472 b	15625.334 a	5025.67 a
IR 78875-176-B-1-B	24824.404 g	840.640 e	20.048 de	86.172 a	12940.651 c	4091.79 bc
IR 79971-B-202-2-4	37201.567 c	1524.386 a	21.158 a	59.944 d	13040.823 c	3806.83 cd
IR 80508-B-194-4-B	22239.397 i	848.389 e	20.304 cd	52.742 e	11443.065 e	3528.29 de
IR 80508-B-194-3-B	48706.175 b	1031.046 c	20.908 ab	55.969 de	12069.889 d	4030.50 bc
IR 79907-B-493-3-1	21168.804 j	935.663 d	20.542 bc	36.088 f	12306.970 d	4843.87 a
IR 81025-B-347-3	58351.239 a	1450.986 b	16.304 h	69.085 c	13413.078 bc	4899.18 a
IR 81025-B-327-3	29211.016 e	710.427 f	20.034 de	60.390 d	13326.433 bc	5085.33 a
NADA	28337.155 f	698.655 f	21.198 a	82.497 ab	10573.764 f	3362.21 e
TAROM	13102.320 l	445.993 h	19.112 g	80.082 b	13805.428 b	4766.62 a

Means in each column, followed by at least one similar letter(s) are not significantly different at 5% probability level using Duncan's Multiple Range Test
I1, I2, I3 and I4 include irrigation regimes of 1, 3, 5 and 7 days, respectively

Further investigation on the interaction of irrigation regimes and genotype (*Table 9*) showed that genotype IR 81025-B-347-3 could achieve the highest central cylindrical area in all irrigation regimes due to genetic reasons; while, Tarom had the lowest central cylinder area in all irrigation regimes and like the previous genotype was in one statistical level in all irrigation regimes. So, in addition to environmental conditions, it can be concluded that the reason of differences between genotypes can be related to internal factors and genetics of studied rice genotypes, which did not correspond to other studies (Akbari et al., 2010; Sadat-jamali et al., 2014) reported that genotypes with less resistance had the lowest central cylindrical area and genotypes with greater resistance had the highest one. Because in the current study, the genotype with least

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resistance had the lowest central cylinder area but another genotype (IR 81025-B-347-3) with low resistance had the highest pollen area (58351.239 μm^2).

Table 9. *Mean comparison of two years related to the anatomy of root, yield and yield components in study treatments*

Irrigation regimes	Genotypes	Central cylindrical area (μm^2)	Xylem area (μm^2)	Weight of one thousand seeds (g)	Fertility rate (%)	Dry matter per hectare (kg/h)	Grain yield (kg/h)
I1	VANDANA	25362.112 l-p	1069.939 e-g	20.558 b-i	69.779 j-m	10595.500 p-u	2332.10 xy
	IR 78908-193-B-3-B	20033.825 r-t	470.008 t-v	18.756 no	95.933 ab	15405.833 bc	4038.01 j-t
	IR 81429-B-31	34068.070 e-g	558.905 r-v	18.609 no	84.707 c-h	15549.234 bc	4551.67 d-n
	IR 78875-176-B-1-B	25214.323 l-p	911.400 g-n	20.221 e-j	78.460 f-j	13769.404 e-h	2949.50 u-x
	IR 79971-B-202-2-4	38285.522 d	1901.032 a	21.055 a-g	91.772 a-e	11846.333 j-p	3541.83 o-w
	IR 80508-B-194-4-B	22871.524 o-r	904.501 g-n	20.609 b-i	79.856 f-j	12518.579 i-l	4487.50 f-p
	IR 80508-B-194-3-B	50647.838 b	1133.497 d-f	20.422 e-i	75.152 g-k	11558.119 k-r	3559.83 n-w
	IR 79907-B-493-3-1	22067.025 p-r	999.127 f-j	20.828 a-h	38.489 qr	11014.058 o-t	4779.17 d-k
	IR 81025-B-347-3	59817.069 a	1779.029 ab	16.039 q	95.275 a-c	12734.312 h-k	4582.17 d-m
	IR 81025-B-327-3	31611.007 g-i	796.763 l-p	19.759 i-m	65.957 k-n	13234.310 f-i	5233.67 c-g
	NADA	29403.344 h-k	817.727 j-p	21.424 a-d	78.572 f-j	10088.007 s-u	3087.83 t-x
	TAROM	13544.244 uv	485.463 t-v	19.161 k-n	92.635 a-d	15517.545 bc	6066.33 a-c
I2	VANDANA	25170.883 l-p	1011.017 f-i	20.998 a-g	58.959 n-o	11207.031 m-s	3531.67 o-w
	IR 78908-193-B-3-B	19648.587 r-t	435.382 t-v	19.278 k-n	66.630 k-n	12918.733 g-j	4479.83 f-p
	IR 81429-B-31	32943.967 f-h	537.236 r-v	19.093 l-n	72.670 i-l	19291.015 a	5361.17 c-f
	IR 78875-176-B-1-B	25116.249 l-p	857.238 i-p	20.690 b-i	91.469 a-e	16032.245 b	5113.33 c-h
	IR 79971-B-202-2-4	37995.980 d	1711.029 b	21.500 a-c	73.628 h-l	13791.627 e-h	4145.50 h-r
	IR 80508-B-194-4-B	22772.810 o-r	850.746 i-p	21.140 a-e	62.756 l-n	10645.846 p-u	4128.01 h-s
	IR 80508-B-194-3-B	50118.397 bc	1062.977 e-h	21.528 ab	68.704 j-n	13803.437 e-h	5398.83 c-f
	IR 79907-B-493-3-1	21938.729 p-r	950.264 f-m	21.222 a-e	46.124 pq	14028.987 d-g	5530.33 b-d
	IR 81025-B-347-3	58902.192 a	1521.026 c	16.995 p	59.972 m-o	15051.500 b-e	6362.55 ab
	IR 81025-B-327-3	30648.724 g-j	754.331 n-q	20.518 c-i	87.215 a-f	14858.255 b-e	6555.10 a
	NADA	29024.263 i-l	760.457 n-q	21.788 a	96.574 ab	11696.049 j-q	4501.50 e-o
	TAROM	13431.251 uv	457.401 t-v	19.975 h-l	95.381 a-c	15267.167 b-d	6024.11 a-c

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I3	VANDANA	23477.248 n-r	988.017 f-k	20.953 a-h	46.425 pq	12200.833 i-o	4379.17 g-p
	IR 78908-193-B-3-B	17945.766 st	416.116 uv	21.013 a-g	86.203 b-g	13405.519 f-i	4628.67 d-l
	IR 81429-B-31	30560.788 g-j	520.457 s-v	20.412 e-i	82.346 d-i	14951.377 b-e	5378.06 c-f
	IR 78875-176-B-1-B	24698.453 m-q	812.578 j-p	20.327 e-j	98.029 a	12398.370 i-m	4978.66 d-j
	IR 79971-B-202-2-4	37012.584 de	1270.463 d	21.502 a-c	49.913 op	14321.500 c-f	3900.83 l-u
	IR 80508-B-194-4-B	21797.306 p-s	835.733 i-p	20.290 e-j	32.242 rs	12744.667 h-k	3628.16 m-v
	IR 80508-B-194-3-B	47310.524 bc	975.877 f-l	21.158 a-e	43.453 pq	12326.167 i-n	4224.33 h-q
	IR 79907-B-493-3-1	20758.809 q-s	914.015 g-n	20.890 a-h	36.294 qr	13854.333 e-h	5405.33 c-f
	IR 81025-B-347-3	57791.145 a	1301.361 d	16.147 q	70.094 j-m	14768.333 c-e	5484.67 b-e
	IR 81025-B-327-3	27993.566 i-m	687.603 p-s	20.447 e-i	46.615 pq	14761.667 c-e	5055.01 d-i
	NADA	27946.195 i-m	615.270 q-t	21.085 a-f	84.555 c-h	10910.500 p-t	3147.67 s-x
	TAROM	12932.878 v	431.748 t-v	19.252 k-n	81.328 e-i	13849.167 e-h	4368.66 g-p
I4	VANDANA	21687.729 p-s	712.423 o-r	20.065 g-k	41.059 p-r	10704.833 p-u	3922.33k-u
	IR 78908-193-B-3-B	16629.824 tu	381.106 v	20.085 f-k	87.355 a-f	11374.609 l-s	4065.67 i-t
	IR 81429-B-31	29948.589 h-k	445.391 t-v	19.823 i-m	82.166 d-i	12709.711 h-k	4811.83 d-k
	IR 78875-176-B-1-B	24268.589 m-q	781.343 m-q	18.955 mn	76.728 f-k	9562.587 u	3325.66 q-w
	IR 79971-B-202-2-4	35512.183 d-f	1215.020 de	20.573 b-i	24.464 s	12203.833 i-o	3639.17 l-v
	IR 80508-B-194-4-B	21515.946 p-s	802.573 k-p	19.178 k-n	36.112 qr	9863.167 tu	1869.50 y
	IR 80508-B-194-3-B	46747.940 c	951.835 f-m	20.525 c-i	36.568 qr	10591.833 p-u	2939.01 u-x
	IR 79907-B-493-3-1	19910.653 r-t	879.244 h-o	19.227 k-n	23.443 s	10330.500 r-u	3660.67 l-v
	IR 81025-B-347-3	56894.552 a	1202.527 de	16.033 q	50.997 op	11098.167 n-t	3167.33 r-x
	IR 81025-B-327-3	26590.768 k-o	603.010 q-u	19.413 j-n	41.773 p-r	10451.500 q-u	3497.67 p-w
	NADA	26974.818 j-n	601.167 q-u	20.493 d-i	70.285 j-m	9600.500 u	2711.83 v-y
	TAROM	12500.909 v	409.359 v	18.060 o	50.984 op	10587.833 p-u	2607.50 w-y

Means in each column, followed by at least one similar letter(s) are not significantly different at 5% probability level using Duncan's Multiple Range Test
I1, I2, I3 and I4 include irrigation regimes of 1, 3, 5 and 7 days, respectively

However, the results corresponded to other studies (Lopes and Reynolds, 2010; Daxylem, 2016) on the reduction of traits size related to root anatomy such as central cylinder, which can reduce vascular bundles area by reducing the interior space in order to increase the efficiency of water (*Table 5*). Positive and significant correlation between central cylindrical area and xylem (0.667**) also showed increased likelihood of positive and negative impacts of these two traits in a line (*Table 10*).

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Table 10. *Correlation coefficients between grain yield and root anatomy traits and yield components of rice genotypes*

	1	2	3	4	5	6
1. Grain yield	1	0.092	-0.020	-0.032	0.249**	0.672**
2. Area of central cylinder		1	0.677**	-0.308**	-0.012	0.083
3. Xylem area			1	-0.067	-0.102	-0.069
4. Weight of one thousand seeds				1	-0.020	-0.080
5. The fertility rate					1	0.292**
6. Dry matter yield						1

* and **: Significant at 5% and 1% probability levels, respectively

Xylem area (posterior) of root

The results of combine analysis showed that there was a statistically significant difference between the year, irrigation regimes and genotypes and interaction of irrigation regimes and genotypes at 1% level and was no significant difference with other factors (*Table 7*). Increasing irrigation intervals had negative effects on the xylem area (*Table 8*). Mean comparison of the interaction of two factors showed that genotypes IR 79971-B-202-2-4 and IR 78908-193-B-3-B had the highest and the lowest xylem areas in all irrigation regimes, respectively due to genetic reasons; like other genotypes, it decreased with increasing irrigation intervals (*Table 9*). Due to the task and nature of xylem vessels in transferring water and nutrients, it seems that more water was maintained in genotype IR 78908-193-B-3-B with high resistance. On the other hand, in the first irrigation regime (1-day interval) with relatively larger vessels (985.616 μm^2), water transfer and drain was faster, which is considered a positive factor in warm conditions of rice growing environment. Given that the movement of water in Xylem vessels is affected by water uptake (xylem vessels wall) or adhesive force in addition to cohesive force; therefore, capillary rise of water in xylem vessels depends on the angle of the liquid contacting the wall, xylem vessels' radius and surface tension of liquids as well as gravity force, because the capillary rise in vessels is inversely proportional with its radius as a result capillary force retaining water is higher with smaller xylem area, and more pressure is needed to move water in vessels, which can be the main cause of compatibility of genotypes with high resistance to drought conditions due to reduced xylem area and hence improving the photosynthetic efficiency and water consumption (Daxylem, 2016; Hamadanzadah et al., 2015). By this reason, water is maintained with more force in genotype IR 78908-193-B-3-B with smaller xylem area (425.653 μm^2). In this system, transpiration is a physical phenomenon to produce movement force for water and inorganic elements (McKenzie et al., 2009; Daxylem, 2016). Given the importance of this route, changes in the xylem area at the time of planting and according to different environmental conditions and the amount of irrigation can disrupt the absorption and transportation processes of water and nutrients to photosynthetic parts (*Figure 2*). Results correspond to Limouchi et al. (2013) about reduced xylem area with increased stresses, drought stress in this study (compared to favorable conditions at heading stage).

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Number of xylem vessels in root

According to *Figures 2* and *3*, number of xylem vessels in root was not affected by irrigation regimes and had a stable trend in all irrigation regimes and differences were due to internal factors and genetic reasons of investigated genotypes. Genotype IR 81025-B-347-3 had the highest number with 8 xylem vessels in cross-section of root. It seems that this genotype could have a consistency to absorb and maintain more water with increased number and decreased area due to lower central cylinder area. The results corresponded to (Daxylem, 2016) that number of xylem vessels depends on internal factors of plants and would not be affected by surrounding environment such as irrigation.

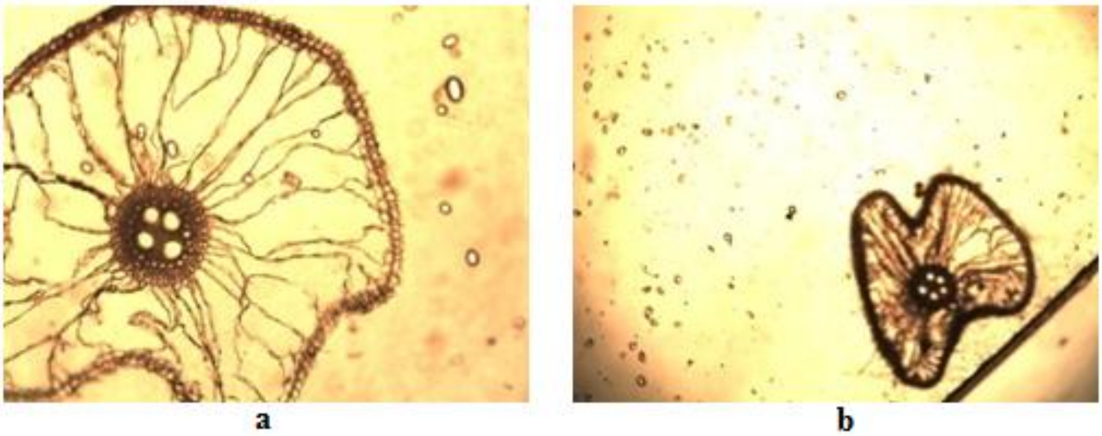

Figure 2. *Root anatomy; a and b: cross-section of the root with a magnification of 40 and 10, respectively*

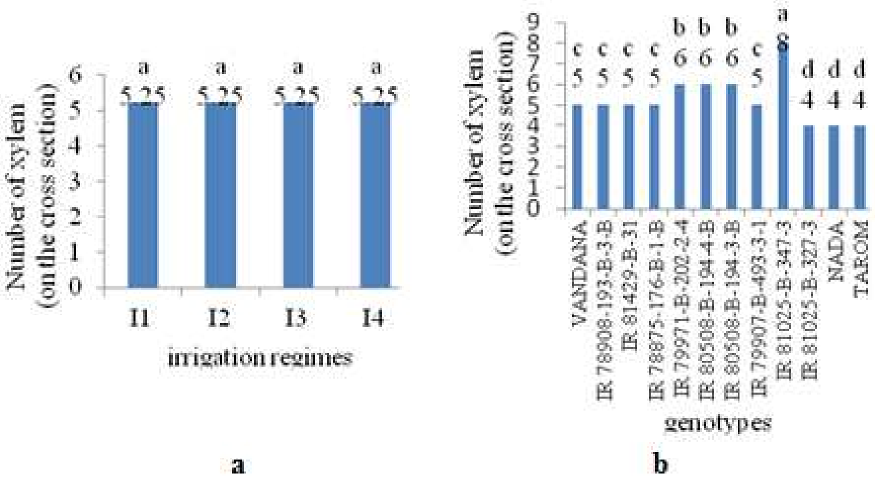

Figure 3. *Number of posterior xylem bundles; a and b: number of vascular bundles in the different irrigation regimes and aerobic rice genotypes, respectively. I1, I2, I3 and I4 include irrigation regimes of 1, 3, 5 and 7 days, respectively*

The weight of one thousand seeds

The results of combined analysis showed that except for the interaction of year and irrigation regimes, other factors and the interaction between them had significant

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differences at 1% (*Table 7*). The highest and lowest weights of one thousand seeds were seen in the second irrigation regime (3-day interval) (20.394 g) and the fourth irrigation regime (7-day interval) (19.369 g), respectively (*Table 8*). As it can be seen, genotypes in 7-day interval irrigation regime had less weight of one thousand seeds in addition to reduced grain yield. The highest and lowest weights of one thousand seeds were achieved for Neda (21.788 g) in the second irrigation regime and genotype IR 81025-B-347-3 (19.033 g), respectively. Increased capacity of active reservoir and more strength to provide assimilates to seed in particular by providing favorable conditions in 3-day interval irrigation regime for genotype Neda contrary to other genotypes can be the reasons for reaching this conclusion. The results corresponded to other researchers (Arvin and Vafabakhsh, 2016) about the relationship between source and reservoir in increased weights of one thousand seeds and increased strength in ideal irrigation to fill the reservoir (*Table 9*).

Fertility rate

The results of this study showed that there was significant difference between years, irrigation regimes and genotype at 5% level and between the interaction of two factors at 1% level, but no difference was observed in other cases (*Table 7*). Mean comparisons represented the different reactions of genotypes to different irrigation regimes; despite the decline in fertility rate from the first to fourth irrigation regimes that actually represented the negative impact of increased drought on this trait. The highest and lowest fertility rate were seen in genotype IR 78875-176-B-1-B (98.029%) in the third irrigation regime (5-day interval) and genotype IR 79907-B-493-3-1 (23.443%) in the fourth irrigation regime (7-day interval), respectively. The result could be due to different responses of genotypes in the processes of production and accumulation of nonstructural carbohydrate soluble in vegetative parts of plants as well as panicle structure and its role in providing photosynthesis material in reproductive and vegetative parts and finally the harvest index. Soluble nonstructural carbohydrate plays an important role in the production of viable pollens, respiration rate and filling rate. It was found that by changing the irrigation regime and increasing fertility rate, the amount of soluble carbohydrates in vegetative parts at the time of physiological maturity had significantly declined (*Tables 8* and *9*). The results corresponded to others (Arvin and Vafabakhsh, 2016; Shanmugasundaram, 2015) about increased sterility due to high drought, the release and fertility of pollen role in fertility rate and the negative effects of high drought during maturity.

Dry matter yield per hectare

The results of this study showed that except for effects of years and genotypes as well as years, genotypes and irrigation regimes, other factors had significant differences. The interaction between years and irrigation regimes had significant differences at 5% level and other factors were significantly different at 1% level (*Table 7*). Relative superiority of dry matter of studied genotypes in the 3-day, 5- day and 7-day interval irrigation regimes and also genotypes with high resistance showed that changes in this trait fit perfectly with the grain yield (*Table 8*). Given that the highest grain yield was obtained in this irrigation regime and genotypes, it can be a result of increased leaf area index and net photosynthesis in order to transfer to the main reservoir (seeds). The highest (19291.015 kg/ha) and lowest (9562.587 kg/ha) dry

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matter per hectare was seen in genotype IR 81429-B-31 with high resistance in the 3-day interval irrigation regime (*Table 2*) and genotype IR 78875-176-B-1-B in the 7-day interval irrigation regime, respectively (*Table 9*). In addition to grain yield, the results can be explained by genetic differences and their different responses to different irrigation regimes. Although the results are not consistent with (Ghasemi-Nasr et al., 2016), which stated flood irrigation increased dry matter, but corresponded to (Abdola and Zarea, 2015) about the reduction of dry matter in 1- day irrigation regime flooding) due to increased nutrients washing and making them unavailable for plants. The results are consistent with (Durand et al., 2016) on the reduction of nutrients accumulation to increase dry matter due to reduced plant growth period In terms of increased drought according to what happened in the fourth irrigation regime (7-day interval).

Grain yield

According to combined analysis results between years, irrigation regimes, their interactions as well as between genotypes and interaction of genotypes and irrigation regimes were significant at 1% level. However, there were no significant differences for the interaction of genotypes and year and the simultaneous effect of three factors and this indicates that grain yield was affected by genetic features, different irrigation regimes and their positive integration (*Table 7*). Mean comparisons showed that the highest grain yield (5094.31 kg/ha) was related to the 3-day interval irrigation regime, which had increased the production compared to flooding irrigation regimes (probably due to the incompatibility and waste of resistance energy such as the energy for creating aerenchyma) and also 5- and 7-day interval irrigation regimes (could be due to the unavailability to nutrients and assimilate accumulation at the base of plants) with rates of 19.50, 10.72 and 34.21%, respectively or 993.52, 546.05 and 1742. 79 kg/ha, respectively (Durand et al., 2016; Mohd Zain and Razi Ismail, 2016). Recent results according to others (Tarlera et al., 2015) indicated that the second irrigation regime and in the absence of water, 5-day interval irrigation regime might be appropriate to increase irrigation efficiency and reducing environmental pollutants such as methane. Besides, in addition to being compatible with most of the studied genotypes, permanent flooding conditions can wash nutrients and make them unavailable for plants. According to the reducing trend from first to fourth irrigation regimes, it seems that different reaction during different developmental stages due to the limited assimilates and shorter filling and grain growth periods are one of the reasons for reaching this conclusion (*Table 8*). Genotypes IR 81025-B-327-3 was superior to the other rice genotypes that the highest value (6555.10 kg/ha) was belonged to the second irrigation regime (3-day interval). Escaping from the water stress by reducing plant height to 10-20 cm, especially in the maturity period, as well as the allocation of more carbohydrates to the main reservoir are the reasons for compatibility and superiority of this genotypes. The interaction of two factors showed that according to other studies (Tavala et al., 2015) genotypes responses to different irrigation regimes regarding their tolerance threshold thus traits related to genotypes was different. Hence, all genotypes in the fourth irrigation regime due to the short growing season and thus Allocation of less carbohydrates and minerals transferred to the main tank and ultimately Reduction of the tank activity and dry matter accumulation capacity (capacity × number of seed), grains had the lowest yield (*Table 9*). The results are consistent with other studies (Durand et al., 2016) on the reduction of grain yield in terms of increased tension, higher than plant tolerance due to the growth disturbance during reproductive stage and lack of

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transferring and allocation of carbohydrates and sugars to the grain as well as other studies (Abdola and Zarea, 2015) based on yield loss in flooded conditions; however, it is not in accordance with study of Ghasemi-Nasr et al. (2016), which stated increasing available water for roots in flooded irrigation had increased rice yield.

The results of regression analysis of grain yield and traits showed that the lengths of stigma and style and xylem area are factors affecting grain yield; so that, 0.016% of the changes associated with 0.01% more than the style length was related to the stigma length, which in turn represents the role and importance of this trait compared to the traits in rice production. According to recent results, in order to achieve an optimum value for this index, creating a perfect reservoir can provide a base for transferring dry matter to the reservoir and the formation of economic performance in line with increased production (*Table 11*). The results corresponded to (Oyiga et al., 2010) about the effect of the stigma on grain yield due to its high influence on receiving pollen and fertilization.

Table 11. *Stepwise regression analysis of grain yield and floret and root anatomical properties based on all the data*

Model	Partial R^2	Model R^2
$Y = 3827.462^{**}+0.455^{*}X_1$	0.016	0.016
$Y = 3981.436^{**}+0.649^{**}X_1-0.956^{*}X_2$	0.016+0.015	0.031
$Y = 4321.589^{**}+0.805^{**}X_1-1.317^{**}X_2-0.420^{ns}X_3$	0.016+0.015+0.01	0.041

^{ns}, * and **: Nonsignificant and significant at 5 and 1% level of probability, respectively
X_1 = the stigma
X_2 = the style
X_3 = xylem area (Posterior)

Conclusion

The results of the present study indicated that the size of anatomical characteristics of florets was more dependent on internal properties of the plant; so that, genotypes with larger and smaller sizes of these traits maintained their preference to other genotypes while under the influence of different irrigation regimes. In general, increasing irrigation intervals from the second (3-day interval) to the fourth (7-day interval) irrigation regimes and thus increased drought around plants reduced pollen area and as a result nutrients reservation was also reduced that can reduce its transfer to ovaries and plants fertility rate. The first irrigation regime with one day rotation also in flood The IR 80508-B-194-4-B genotype had the highest grain pollen area in all irrigation regimes due to genetic reasons. Increasing the irrigation interval had a negative effect on the pollen grain surface due to reduced food and energy needed to reach ovaries that ultimately led to lack of fertility and reduced grain yield conditions, due to the aerobic nature of genotypes, reduced pollen grains due to more energy to expand the adaptive mechanisms such as arranchym. From the highest to the shortest anther length, the irrigation regimes were observed in intervals of five, seven, three and one days, respectively. The IR 78908-193-B-3-B genotype also had the highest anther length among all irrigation regimes, except for a significant decrease in the second irrigation regimen with a three-day rotation that could be a major factor in increasing fertility and grain yield was due to the proximity to the stigma and hence more effective grain

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transfer, but no significant difference was observed in other irrigation regimes. In the fourth irrigation regimen with a seven-day irrigation interval, we observed a decrease in anther width, but in other irrigation regimes there was no difference in width. The IR 80508-B-194-3-B genotype had the highest anther width in all studied irrigation regimes, which increased the irrigation intervals and, as a result, drought reduced. The highest length of the stigma was for IR 78875-176-B-1-B genotype in all irrigation regimes, which, like other genotypes, increased with increasing length of irrigation intervals. The length of the style in the fourth irrigation regimen with a seven-day interval decreased, and there was no significant difference in other irrigation regimes. The IR 78908-193-B-3-B genotype also had the highest length of style in all irrigation regimes, which increased the length of style by increasing irrigation intervals, so that it had the maximum length of style in the fourth irrigation regimen with irrigation interval of seven on the contrary to genotypes with less resistance and therefore more favorable compatibility with these conditions. Due to having a good-sized stigma, style, anther and anther width, the second irrigation regime was able to get and keep more pollen and thus increased fertility rate and grain yield. While in other irrigation regimes such as the fourth irrigation regime (7-day interval) with increased anthers and reduced style length, the distance between male and female organs increased and thus receiving pollen decreased. The results also showed water shortage in 5- and 7-day irrigation regimes caused a reduction in central cylindrical area and xylem area, which could be a mechanism to increase water use efficiency and reduce the transpiration. The results showed that the IR 81025-B-347-3 and IR 79971-B-202-2-4 genotypes had the highest values for all the irrigation regimes in the central cylindrical and xylem area crests, respectively. By increasing the irrigation intervals and thus the drought around the plant to adapt to the conditions and increase the efficiency of use, the potential and the transfer of water from the surface were reduced. As a result, the dryness around the plant was reduced to adapt to the conditions and to increase the efficiency of use, the potential and the transfer of water from their surface. Of course, it could lead to a reduction of material transferring and ultimately grain yield such as that obtained for the fourth irrigation regime (7-day interval). Among the traits dependent on yield and grain yield components, weight of a thousand seeds dry matter and seed yield were highest to the lowest in irrigation regimes of three, five, one and four days, respectively. Fertility also increased the irrigation intervals due to reduced pollen grains and lack of energy to reach the ovary and to inoculate. NADA genotype had the highest weight of a thousand seeds in irrigation regimes of one and three days and the IR 79971-B-202-2-4 genotype had the highest amount in irrigation regimes of five and seven days. Increasing the weight of a thousand seeds in low-resistance genotypes in conditions of increasing irrigation intervals over the previous discussion could result in a reduction in the number of florets and more infertility, thereby reducing competition and increasing the space in the paniculum. The IR 78908-193-B-3-B genotype had the highest fertility rates in first and fourth irrigation regimes, with one and four-day irrigation intervals, NADA and IR 78875-176-B-1-B genotypes also had the highest fertility rates, respectively in the second and third irrigation regimes with irrigation intervals of three and five days according to the previous descriptions. Due to the large canopy structure, large leaves and thick stems, IR 81429-B-31 genotype with the highest dry resistance had the highest dry matter yield in all irrigation regimes. The TAROM, IR 81025-B-327-3, IR 81025-B-347-3 and IR 81429-B-31 genotypes have the highest grain yield in irrigation regimes of one, three, five and seven days, respectively. Resistant genotypes

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with improved resistance to irrigation intervals maintained their grain yield better than less resistant genotypes. However, under daily irrigation conditions due to increased energy, resistance to other genotypes had lower grain yield, which could be used depending on the different moisture conditions. The results of this study indicated that the second irrigation regimen with three-day irrigation interval was able to adapt the aerobic rice genotypes with the highest positive and significant correlation with grain yield and decrease the negative correlation traits. Finally, the relative yield of genotypes was increased. The IR 81025-B-327-3 genotype with an average of 10.6555 kg/h in the second irrigation regimen had the highest grain yield and could be recommended as the superior genotype for cultivation in similar conditions.

Therefore, it can be suggestions for further studies that by focusing main goals on reducing the central cylinder area and xylem of root to increase water use efficiency in dry condition and controlling lengths of florets parts we can hop to increase connection between pollen and flower and finally fertility to increase production under different irrigations.

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DIFFERENCES IN NITROGEN AND PHOSPHORUS UPTAKE AND YIELD COMPONENTS BETWEEN BARLEY CULTIVARS GROWN UNDER ARBUSCULAR MYCORRHIZAL FUNGUS AND *PSEUDOMONAS* STRAINS CO-INOCULATION IN RAINFED CONDITION

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Abstract. Drought is a major limiting factor of plant production in arid and semi-arid regions including Iran. The use of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria (PGPR) may constitute a biological alternative to increase crop yield and plant resistance in water stress conditions. In order to evaluate the synergistic effect of four strains of *Pseudomonas* and arbuscular mycorrhizal fungi (AMF) on nitrogen and phosphorus uptake and yield components of Iranian barley cultivars (Dasht and Sahra) under rain-fed conditions, a two-year experiment was conducted in factorial randomized complete block design. The results demonstrated that in most of the characteristics, the interaction effect of *Pseudomonas* × AMF on cultivars in both years was significant. While *Pseudomonas* strains exhibited different reactions to cultivar and AMF use, in most of the traits measured, they were superior when compared to control. In both cultivars, the maximum yield parameters studied was obtained from co-inoculation of AMF + S4 strain. The Sahra has more production sustainability in comparison to Dasht (seed yield of Dasht 1293 and 1912 kg/ha; Sahra 1752 and 1939 kg/ha in first and second year, respectively). Moreover, in both years and in both cultivars, the co-inoculation increased P and N concentration and uptake significantly. It increased seed yield, while the yield increase was more evident in the Sahra. According to the results obtained, to achieve the maximum seed yield in rain-fed conditions of Iran, cultivation of Sahra alongside co-inoculation of AMF + *Pseudomonas putida* S4 strain is recommended.
Keywords: *Glamus intraradices; N concentration; P concentration; seed yield*

Introduction

Drought is one of the most serious problems in the Middle East, especially in arid and semiarid countries (Kheiri et al., 2017). Considering population growth rates and limited global water resources, it is anticipated that food security will become a serious challenge in the coming decades (Sheffield et al., 2012). The agricultural sector in Iran is one of the most important economic sectors of the country, and water is the most limiting factor for production (Schoppach et al., 2017). Whereas around 92% of agro products depend on water, less than one-third of the cultivated area is irrigated; the rest is devoted to dry farming (FAO, 2014). In these areas, barley (*Hordeum vulgare* L.) is

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grown mainly under rainfed conditions and could fall victim to drought at any stage of growth (Dijkman et al., 2017; Schoppach et al., 2017).

Extensive research is being carried out throughout the world to develop strategies to cope with drought stress by developing drought tolerant cultivars (Oukarroum et al., 2009), shifting crop calendars (Lu et al., 2017), and optimizing fertilizer use (Cetin and Akinci, 2015), agronomic practices (Mupangwa et al., 2012) and resource management practices (Parry et al., 2005; Habiba et al., 2012). Most of these technologies are cost-intensive. Non-normative and long-term use of chemical fertilizers gradually decreases soil quality, reduces the quality of the product, causes imbalances in the natural ecosystems and extends environmental pollution (Ali et al., 2005; Vurukonda et al., 2016).

As a relatively simple and low-cost alternative strategy, the use of free-living plant growth-promoting rhizobacteria (PGPR) is a promising broad-spectrum means of improving plant growth (Yang et al., 2009). The use of microorganisms, especially for intensive culture and in nutrient-poor soil, is essential to preserving the quality of the soil in Iran (Arzanesh et al., 2011). Recent studies in Iran indicate that microorganisms can also help plants to cope with drought stress (Tabatabaei et al., 2016; Khalilzadeh et al., 2016; Delshadi et al., 2017). These bacteria contribute to plant growth by fixing free-living nitrogen, producing growth metabolites such as plant hormones, increasing the solubility of insoluble compounds such as P and K by producing mineral and organic acids and by siderophore production. This increases the supply of micronutrients, especially iron, and reduces the negative effects of stress ethylene and the production of amino cyclopropane-1-carboxylate (ACC) (Arshad et al., 2008). A large number of PGPRs hydrolyze ACC to ammonia, ethylene and alpha ketobutyrate to prevent overproduction of stress ethylene in plants and reduce root growth by producing ACC deaminase enzyme (Glick, 2014).

Pseudomonas fluorescent has been investigated because of its catabolic versatility, excellent colonization ability at the root surface and the ability to produce a wide range of enzymes and metabolites that may be useful in a variety of biotic and abiotic stresses (Mayak et al., 2004). *Pseudomonas* is an important ACC deaminase enzyme-producing bacteria (Glick, 2014). In many arid regions of the world and in Iran, it reduces the negative effects caused by stress tests. Arshad et al. (2008) demonstrated that inoculation of wheat (*Triticum aestivum*) with *Pseudomonas putida* under drought stress improved water status and an additional elastic adjustment that increased grain yield and mineral quality (Mg, K, and Ca). Frohlich et al. (2012) carried out greenhouse and field experiments and reported that *Pseudomonas* sp. DSMZ 13134-treated barley showed increased yield and straw weight under nutrient deprivation. Plants treated with exopolysaccharide (EPS) producing bacteria displayed increased resistance to water stress (Bensalim et al., 1998).

The EPS-producing strain of *Pseudomonas putida* GAP-P45 formed a biofilm on the root surface of sunflower seedlings and imparted tolerance to plants against drought stress (Sandhya et al., 2009). The inoculated seedlings showed improved soil aggregation and root-adhering soil and higher relative water content (RWC) in the leaves. Inoculation of two different crops (tomatoes and peppers) with PGPR *Pseudomonas putida* KT2440 augmented the antioxidant catalase under severe drought conditions, suggesting that trehalose produced by these microorganisms correlates with their ability to protect plants against drought (Vílchez et al., 2016).

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Arbuscular mycorrhiza fungi (AMF) form beneficial symbiotic associations with most plants and play a vital role in plant growth under various conditions by modifying the root system and enhancing the mobilization and uptake of several essential elements. They have also been reported to stimulate plant stress tolerance by enhancing enzymatic as well as non-enzymatic antioxidant defense systems (Wu et al., 2014; Ahmad et al., 2014), lipid peroxidation (Abd_Allah et al., 2015), and phytohormone synthesis (Navarro et al., 2013).

Researchers have stated that some *Pseudomonas* strains can have stimulatory effects on AM growth under drought stress (Meyer and Linderman, 1986). One study found that severe colonization of barley roots by AMF increases plant growth and dry matter production (Chaurasia and Khare, 2005). AMF helper bacteria colonization such as *Pseudomonas* stick to root surfaces and reform the symbiotic relationship between AMF and the plant to provide many benefits to the host plant (Schalamuk et al., 2006). AMF helper bacteria offer mechanisms that affect the deployment and performance of AMF symbiosis and stimulates spore germination, fungal contact with roots or prompt AMF root production (Frey-Klett et al., 2007; Parmar and Dufresne, 2011; Nadeem et al., 2014).

These findings indicate that the co-inoculation of AM and *Pseudomonas* can enhance the activity of AM during symbiosis with the host plant (Artursson et al. 2006). Barea et al. (2005) concluded that the cooperation of *Pseudomonas* and AM in nutrient uptake by plants is probably due to specific attributes of the microorganisms and there is a growing interest in improving understanding of their involvement in nutrient cycling and non-nutritional physiological values that make the plant more tolerant of drought stress.

There have been no investigations thus far into the synergistic effect of root colonization of fluorescent Pseudomonads and AMF on barley under dry land farming conditions in Iran. In many crops, root colonization by fluorescent Pseudomonads and AMF changes a plant's nutrient uptake, growth and drought resistance. The present study focused on the agronomic effects of native *Pseudomonas* strains and AMF on N and P uptake and yield in barley. The hypothesis tested was that the co-inoculation of *Pseudomonas* strains and AMF would increase the nutrient uptake and yield of barley cultivars and would induce resistance against water deficit.

Materials and methods

The study site

A field experiment was carried out in Research Center of Ardebil Islamic Azad University with a height of 1460 meters above sea level, longitude 48˚20' and latitude of 38˚19' under rain-fed conditions during both 2014-2015 and 2015-2016 growing seasons. Climatic condition of Ardebil province is characterized by a semi-arid climate (humid winters and dry summers). Rainfall distribution is fluctuate in Ardebil province and most of precipitation occurs between October and May, and precipitation of long term is 424 mm. Annual rainfall were 348 and 407 mm during 2014-2015 and 2015-2016 growing seasons respectively in Ardebil. Mean monthly rainfall (*Figure 1*) and monthly temperature (*Figure 2*) during plant growth for both years have been presented. Soil samples were collected from the surface (0-30 and 30-60 cm depth) before sowing. Samples were air dried and powdered with wooden mortar and pestle and passed through a 2 mm stainless steel sieve. Soil experiment results are presented in *Table 1*.

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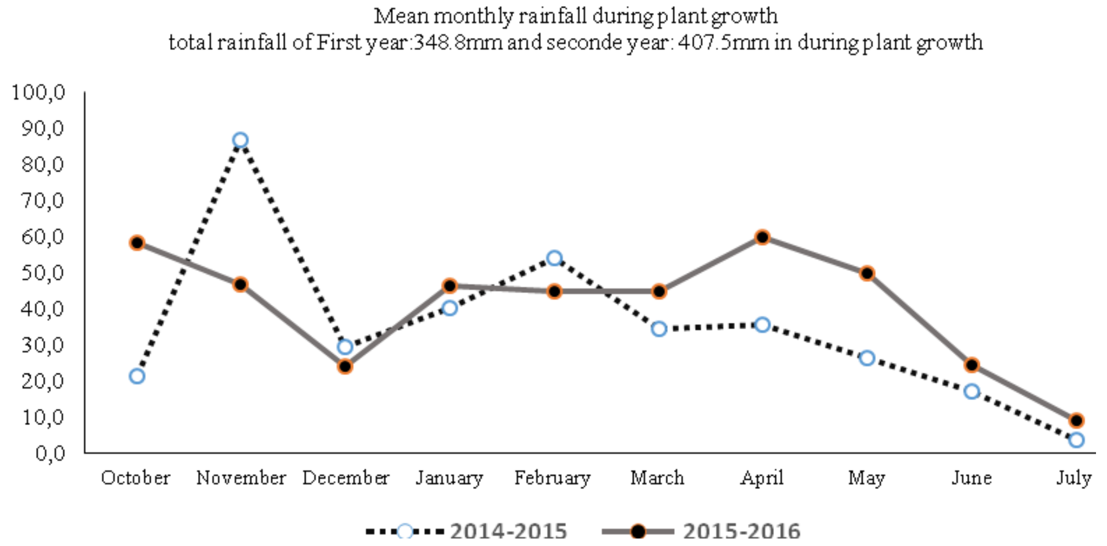

Figure 1. *Mean monthly rainfall during the growth period of plant (Source: Weather Station at Experimental Farm of Azad University of Ardabil)*

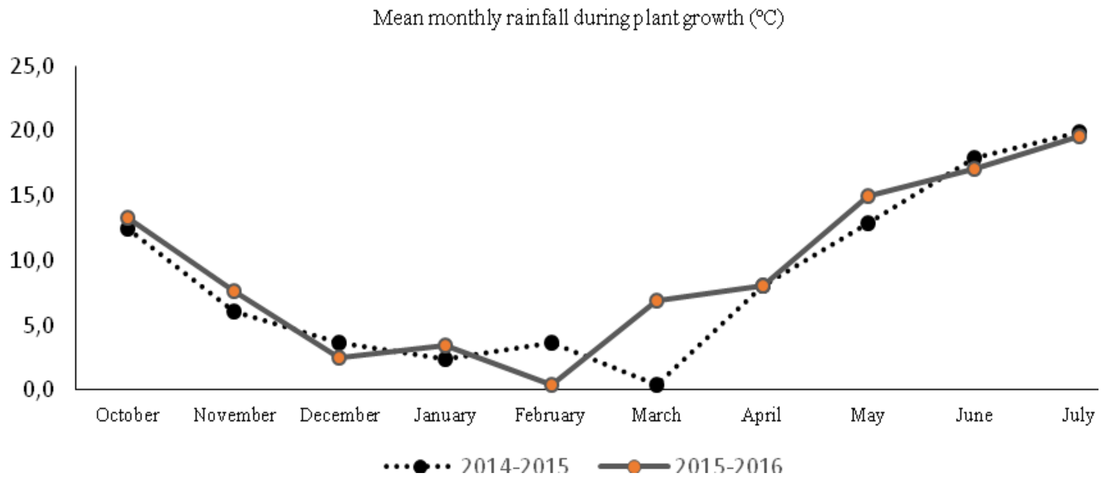

Figure 2. *Mean monthly temperature during the growth period of plant (Source: Weather Station at Experimental Farm of Azad University of Ardabil)*

Table 1. *Soil Physical and chemical characteristics of the soil during the two-year study*

Years	Depth (cm)	Sand	Silt	Clay	Ec	pH	Organic Carbon	N	P	K	Zn	Fe	Mn
			(%)		(dS/m)		(%)	(%)				(mg/kg)	
2014-15	0-30	26	42	32	0.83	7.3	0.8	0.08	8.2	188	0.52	3.9	9.12
	30-60	26	31	43	1.04	7.3	0.51	0.05	7.5	156	0.31	2.9	6.2
2015-16	0-30	29	35	36	1.10	7.4	0.82	0.07	9	155	0.8	5.8	10.6
	30-60	27	29	44	1.60	7.3	0.61	0.066	6.6	131	0.4	4.6	6.6

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Treatments and experimental design

Experiment was conducted in a factorial randomized complete block design with three replications. Experimental factors include AMF species *Glamus intraradices* strain of BEG11 (inoculated (M1) and non-inoculated (M0)), strains of *Pseudomonas* (four strains of *Pseudomonas* bacteria, including S153, S169, S4, S8 and a control (without inoculation) and barley cultivars (Dasht and Sahra cultivars).

Preparation and characterization of Pseudomonas strains

These *Pseudomonas* strains were isolated from soil samples collected from fields in Ardebil province, Iran. The strains were evaluated for their siderophores, P-solubilizing capability, acid and alkaline pH, and produces the phytohormone indole-3-acetic acid (IAA) (Alishahi et al., 2013). *Pseudomonas* strains was characterized as described below:

Siderophore production by *Pseudomonas* strains were tested on universal Chrome Azurol S (CAS) agar (Schwyn and Neilands, 1987). The bacterial strain was inoculated at the center of the plate and incubated at 28 °C for 3 days. Siderophores production were detected by a halo of color change from blue to orange on the CAS medium and measured in triplicate as the ratio between the two diameters of the halo and the two colony diameters.

Phosphate solubilization by *Pseudomonas* strains were assayed according to Goldstein (1986), using two different media, one containing dicalcium phosphate (DCP) (NH_4Cl 4.25 g l^{-1}, NaCl 0.85 g l^{-1}, glucose 8.5 g l^{-1}, $MgSO_4$ $7H_2O$ 0.85 g l^{-1}, K_2HPO_4 2 g l^{-1}, $CaCl_2$ $2H_2O$ 4 g l^{-1}, agar 17 g l^{-1}) and one containing tricalcium phosphate (TCP) (NH_4Cl 5 g l^{-1}, NaCl 1 g l^{-1}, $MgSO_4$ $7H_2O$ 1 g l^{-1}, glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ 40 g l^{-1}, agar 20 g l^{-1}). The strains were inoculated at the center of the plate and incubated at 28 °C for 15 days. DCP solubilization was indicated by a clarification halo around the colony; TCP solubilization was identified by a colony growth on the media.

Indole-3-acetic acid (IAA) production by *Pseudomonas* strains were quantified according to De Brito Alvarez et al. (1995). The bacterial strain was inoculated onto a nitrocellulose disk placed on trypticase soy agar (TSA) with 10 % added to L-tryptophan (5mM) and incubated at 28 °C for 3 days. The membrane was then stained with the Salkowskys reagent ($FeCl_3$ 2 % in percloric acid 35 %), the presence of a red/pink halo around the colony indicated a positive reaction.

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme activity was assayed based on Penrose and Glick (2001) method which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The µmole quantity of α-ketobutyrate produced by this reaction was determined by comparing the absorbance of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 nmol at 540 nm. A stock solution of α- ketobutyrate was prepared in 0.1 M Tris-HCl (pH 8.5) and stored at 4o °C. In order to measure the specific activity of the cultures, protein estimation was carried out according to Lowry et al. (1951). *Pseudomonas* strains characterized are presented in *Table 2*.

Table 2. *Characteristics of Pseudomonads strains*

Pseudomonas Strains	Phosphorus solubilizing activity	ACC-deaminase activity µmoles mg^{-1} h^{-1}	IAA production (mg L^{-1})	Siderophore production (halo diameter/colony diameter)
P. fluorescens strain 153	+	4.61	3.1	1.6
P. fluorescens strain 169	+	4.45	2.8	1.5
P. putida strain 4	+	8.17	6.6	1.7
P. putida strain 8	+	7.34	5.9	1.6

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The bacterial strains were kept in nutrient broth with 15% glycerol at –80 °C for long-term storage. For this experiment, a single colony was transferred to 500 mL flasks containing NBRIP and grown aerobically on a rotating shaker for 48 h at 27 °C and 150 rpm. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10^8 colony forming units (CFU) mL^{-1}, and the resulting suspensions were used for the experiment.

Experimental plots, seed inoculation and sowing

Before sowing, barley seeds were soaked by Arabic gum and active bacteria were added to seeds. After inoculation, seeds were dried on a surface sterilized plastic sheet in laminar flow.

The AMF inoculum contained colonized root fragments, sand, AMF hyphae and spores. The inoculum was blended with an inert material for solution and homogenizing the distribution in the soil. A 50-g portion of inoculum was added to each plot at sowing time just below the seeds (Vazquez et al., 2002). The AMF was obtained from the culture collection of the Soil and Water Research Institute (SWRI), Iran.

The first effective rainfall was in November 23 and cultivation operation began after it. In the fall and before planting, plowing operation and disc were conducted and after land leveling, the seeds were planted with density of 400 seeds per m^2. Plots were 6 rows, 5 meters long, and spaced 20 cm. Hand weeding was operated after planting. Farm management was conducted conforming to organic farming principles with no application of chemical materials.

Sampling of mycorrhizal colonization and plant height, yield components, seed yield, and biological yield

The mycorrhizal colonization was enumerated using the method of Philips and Hayman (1970). The roots were examined under a microscope and the percent of root colonization was determined by dividing the number of colonized roots by the number of total roots examined.

In physiological maturity stage, sampling of middle lines of each plot was conducted with respect to the sidelines and among competing bushes. To calculate the plant height, number of fertile tiller per plant, seeds per spike and thousand kernel weight (weight of 1000 seeds), samples taken from 10 plants were randomly selected and counted in each of 10 plants. Then, their mean was calculated in each plant. Seed yield was measured from middle rows of each experiment plot with 1 m^2 area. For measuring biological yield, the samples were dried for 72 h at 70 °C in an oven. Harvest index was calculated by following formula:

$$\text{Harvest index (\%)} = \frac{\text{Seed yield (kg/ha)}}{\text{Biologicalyield (kg/ha)}} \times 100 \qquad \text{(Eq.1)}$$

Nutrient analysis

The seed nitrogen (N) content was determined following the semi-micro Kjedahl procedure using a nitrogen analyzer (Kjedahl, 2300; FOSS, Hoganas, Sweden). The phosphorus (P) content was estimated using the vanadomolybdo-phosphoric colorimetric method (Jackson, 1962). Uptake of seed elements was calculated by following formula:

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$$\text{Element uptake (kg/ha)} = \frac{\text{Seed yield (kg/ha)} \times \text{Element concentration (\%)}}{100} \qquad \text{(Eq.2)}$$

Statistical analysis

Experimental data were analyzed statistically using ANOVA. Significance of the effect of treatment was determined by the magnitude of the F-value ($P \leq 0.05$). When a significant F-test was obtained for the treatments, separation of means were done using the LSMEANS procedure with LSD adjustment at $P = 0.05$. Statistical analysis of the results was performed using general linear model (GLM) in SAS software version 9.2.

Results and Discussion

Plant height

The results demonstrated that the interaction effect of *Pseudomonas* × AMF × cultivars × year had significant on plant height was significant (P<0.05) (*Table 3*). Mean comparison showed that in the first year in the Sahra cultivar at the AMF inoculation levels, there was no significant difference between the *Pseudomonas* strains. Nevertheless, in the Sahra at the AMF non-use conditions, control (76.96 cm) exhibited the greatest height.

In the second year, in both cultivars, S4 strain in comparison to other *Pseudomonas* strains produced more height (0.7-29.5% and 1.1-16.5% under AMF use and non-use conditions, respectively), although there was no significant difference between some strains and control (*Table 4*). Increasing the plant height plays an important role in the remobilization of photosynthetic materials to seeds during seed filling period (Sharma et al., 2014). Vazquez et al. (2000) on their investigation on wheat also reported that AMF and cultivar had significant effect on plant height, but the effect of bacteria on plant height was not statistically significant.

The useful impact of AMF in uptake of water and elements required by plant through root and root development and increased uptake of elements in the soil to be used by plant, which caused increased in height. Roesti et al. (2006) reported that PGPR strains and AMF can synergistically improve the height of wheat without negatively affecting AMF growth. We also found that the impact of *Pseudomonas* strains on plant height was stronger in AMF-treated plants than untreated plants. This result was in line with the result of the study performed by Chaurasia and Khare (2005).

The number of tiller and number of seed per spike

The interaction effect of *Pseudomonas* × AMF × cultivars × year on number of tiller was significant (P<0.05) (*Table 3*). Mean comparison of the number of tillers showed that in the first year in the Dasht cultivar, though *Pseudomonas* strains were superior compared to control, significant differences were not found between strains. Nevertheless, in Sahra cultivar, both in conditions of consumption and non-AMF conditions, strains of bacteria were superior to the control (10-54% and 5-46% under AMF use and non-use conditions, respectively), while this superiority in some strains was not significant and strain S4 had significant superiority compared to other strains (5.5-32% and 12-28% under AMF use and non-use conditions, respectively).

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In the second year, in both cultivars, in the presence and absence of AMF, *Pseudomonas* strains compared to control had significant superiority (16.1-55.2% and 3.3-46% under AMF use and non-use conditions, respectively) and strain S4 compared to other strains had significant superiority (7.0-13.3% and 5.1-29.6% under AMF use and non-use conditions, respectively) (*Table 4*). In total, in both cultivars, application of AMF led to increased influence of *Pseudomonas* strains and the number of tillers in plant.

The interaction effect of *Pseudomonas* × AMF × cultivars × year on seed per spike was significant ($P<0.05$) (*Table 3*). Comparison of the mean interaction effect of AMF and *Pseudomonas* strains inoculation with barley cultivars on seed number in the spike also revealed that in the first year in the Sahra cultivar in AMF non-use conditions, there was no significant difference between *Pseudomonas* strains and control, and at the AMF use conditions, only strain S4 had superiority compared to control (25.7%). In the Sahra cultivar in AMF non-use conditions, with the exception of strain S4, other strains did not demonstrate any significant difference compared to control. Nevertheless, the use of AMF in plants inoculated with *Pseudomonas* strains increased the number of seeds per spike significantly compared to control (29.0-41.9%). In the second year, in the Dasht cultivar, *Pseudomonas* strains showed significant superiority compared to control (8.9-31.3% and 15.2-23.6% under AMF use and non-use conditions, respectively), however in the AMF non-use conditions, there was no significant difference between the strains of *Pseudomonas*. In the Sahra cultivar, similar trend was observed, while in the AMF use conditions, significant difference was not seen between the S169 strain and control. In total, in both years and cultivars, the maximum number of seeds per spike was obtained from S4 strain under the use of AMF (*Table 4*). Given that tillers are determined earlier than flowering stage, inoculation of seeds with growth stimulating bacteria increased the number of fertile tiller.

Table 3. *Analysis of variance effect of barley cultivars under AMF and Pseudomonas inoculation on plant height, tiller number, seed number, thousand kernel weight, biological yield, seed yield and harvest index*

S.O.V	df	Mean Square						
		Plant height	Fertile tiller number per plant	Seed number per spike	Thousand kernel weight	Biological yield	Seed yield	Harvest index
Y (Year)	1	1266**	0.675*	110.5**	508.4**	28256107**	2935321**	0.595ns
Year / Rep	4	99.31*	0.2067ns	215.5**	6.46ns	936534**	418409**	50.72ns
Cultivar (C)	1	20.17ns	1.012**	708.5**	33.2ns	815925*	1445468**	2548**
AMF (M)	1	13.87ns	0.799*	6.912ns	139.5**	160235ns	53093.ns	33.40ns
Bacteria (B)	4	95.02*	0.853**	24.75ns	143.4**	179551ns	129393**	23.41ns
Y × C	1	17.48ns	10.19**	3.201ns	52.53ns	1253585.**	441.90ns	1276**
Y × M	1	6.44ns	0.935*	6.912ns	9.78ns	719975*	18355.ns	156.65**
Y × B	4	33.65ns	2.201**	68.29*	223.56**	379174ns	72103**	65.00*
C × M	1	0.027ns	0.257ns	25.76ns	11.40ns	453870ns	394.5ns	78.44*
C × B	4	42.76ns	0.191ns	73.33*	9.53ns	262673ns	33745ns	42.28ns
M × B	4	92.92*	0.903**	8.03ns	38.90ns	357051ns	31251ns	133**
M × B × C	4	34.25ns	1.113**	2.60ns	24.31ns	441103*	58164**	80.68**
Y× C × M	1	92.22ns	0.00001ns	2.24ns	32.24ns	28830ns	0.049ns	0.137ns
Y × C × B	4	129.7**	0.146ns	116.9**	6.31ns	268825ns	45489*	55.45*
Y × M × B	4	15.37ns	0.863*	58.00*	16.62ns	263291ns	45088*	106.1**
Y × C × M×B	4	60.48*	0.584*	81.47*	30.09ns	311117*	46415**	59.79**
Error	76	30.95	0.136	13.98	18.19	160978	15949	21.77
CV (%)		9.06	9.77	13.56	8.35	12.13	8.33	12.52

**, ** Significant at P < 0.05 and P < 0.01, respectively. CV: Coefficient of Variation*

In both years, some *Pseudomonas* strains enhanced grain number under AMF use through producing more tillers. In this respect, Shaharoona et al (2008) reported an increase in the number of tillers per plant inoculated with growth promoting bacteria.

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Bacteria used in this experiment can produce ACC deaminase enzyme and this increase in number of tiller can be attributed to this enzyme.

Results of this experiment are in line with results of Nadeem et al. (2007) that reported increased number of tillers in rainfed conditions by growth promoting bacteria. Bacteria mediated changes in the elasticity of the root cell membranes is one of the first steps towards enhanced tolerance to water deficiency (Dimkpa et al., 2009). PGPR improves the stability of plant cell membranes by activating the antioxidant defense system, enhancing drought tolerance in plants (Gusain et al., 2015).

Wagar et al. (2004) reported the effect of AMF in increasing the efficiency of *pseudomonas* bacteria. Therefore, depending on the strain combination, microbial interactions within AMF had positive effects on inoculant establishment in roots and resulted in enhanced tillers and number of seeds as compared to single inoculation.

Table 4. *Interaction effect of Pseudomonas strains × AMF × Barley cultivar on plant height, seed per spike and tiller per plant*

Barley cultivars	AMF	*Pseudomonas* strains	Plant height (cm)		Seed number per spike		Fertile tiller number per plant	
			Y1	Y2	Y1	Y2	Y1	Y2
Dasht	M0	Control	73.16a	50.10b	19.36a	23.00b	3.46a	2.77d
		S153	70.96a	55.76ab	21.00a	28.33a	4.36a	3.41c
		S169	74.63a	49.06b	19.50a	28.33a	4.16a	3.65bc
		S4	75.73a	49.36b	19.50a	26.66a	4.20a	4.00a
		S8	73.70a	58.53a	20.06a	28.00a	4.13a	3.79ab
Dasht	M1	Control	69.90a	48.53ab	20.70b	22.333d	3.66a	2.84c
		S153	71.46a	58.03a	22.43b	26.000b	3.93a	3.82b
		S169	74.06a	57.03ab	21.63b	24.333c	4.23a	4.10ab
		S4	72.96a	60.03a	26.03a	29.333a	4.36a	4.41a
		S8	69.80a	42.10b	20.83b	26.333b	4.33a	4.00ab
Sahra	M0	Control	76.96a	51.86a	31.80b	26.00b	2.66b	3.10d
		S153	69.76ab	50.43a	30.13b	31.33a	2.80b	3.57c
		S169	65.66b	52.13a	28.80b	30.66a	3.43ab	4.23b
		S4	73.80ab	54.72a	36.90a	31.33a	3.90a	4.56a
		S8	72.80ab	54.36a	31.43b	31.66a	3.43ab	3.20cd
Sahra	M1	Control	64.93c	44.20b	25.03c	28.00c	2.56c	3.65b
		S153	79.06a	46.80ab	34.83ab	33.00ab	3.23bc	4.25ab
		S169	73.40abc	48.53ab	34.43ab	29.00c	2.83c	4.25ab
		S4	69.86bc	54.86a	35.53a	34.66a	4.20a	4.78a
		S8	79.56ab	54.80a	32.30b	32.33b	3.96ab	4.49ab

Means within a column followed by the same letter are not significantly (P < 0.05) different according to LSD test. (Y1 = 2014-2015; Y2 = 2015-2016) (M0 = AMF non-use; M1 = AMF use)

Thousand kernel weight

Results of analysis of variance revealed that the main effect of AMF and interaction effect of bacteria × year on seed weight was significant ($P<0.01$) (*Table 3*). Mean comparisons of AMF impact on thousand kernel weight revealed that use of AMF increased thousand kernel weight by 11.2% compared to non-use of AMF (*Figure 3*). AMF provided more water to plant by increasing uptake level, thus during the seed filling, it can help the plant and increase the weight of seeds (Estrada-Campuzano et al., 2012). A comparison of the mean interaction effect of bacteria × year also revealed that in both years, the control in comparison to *Pseudomonas* strains was superior (*Figure*

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4). Thousand kernel low weight in rainfed conditions is due to competition of seeds in obtaining food and reduced carbohydrate stored by plant such that number of cells produced decreases and thousand kernel weight decreases, while AMF played a significant role in increasing weight of wheat seeds (Arshad et al., 2008). Allahverdiyev et al. (2015) reported that in other crops such as wheat, inverse relationship was observed between yield elements, such that with changes in yield elements, the production amount cannot be increased from the final range. It seems that increasing adequate water supply from the beginning of plant growth leads to increased tillering and number of leaf. Generally, it leads to increased yield elements and thus seed yield.

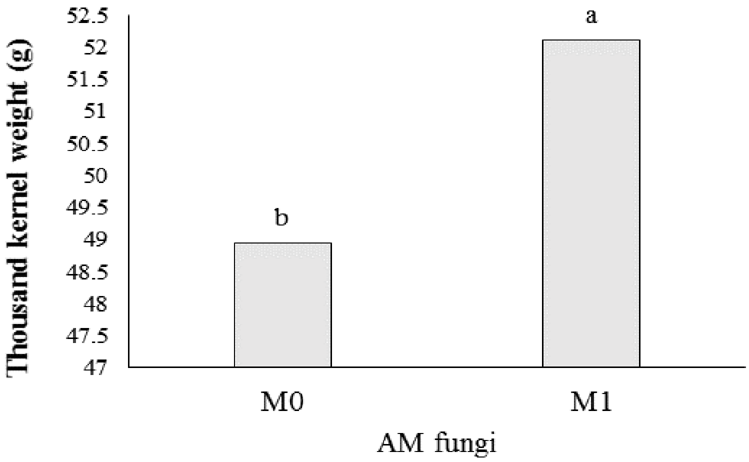

Figure 3. *Main effect of AMF on thousand kernel weight*
M0 = AMF non-use; M1 = AMF use; Means in each column followed by at least one similar letter are not significantly different at 5% probability level, using LSD test

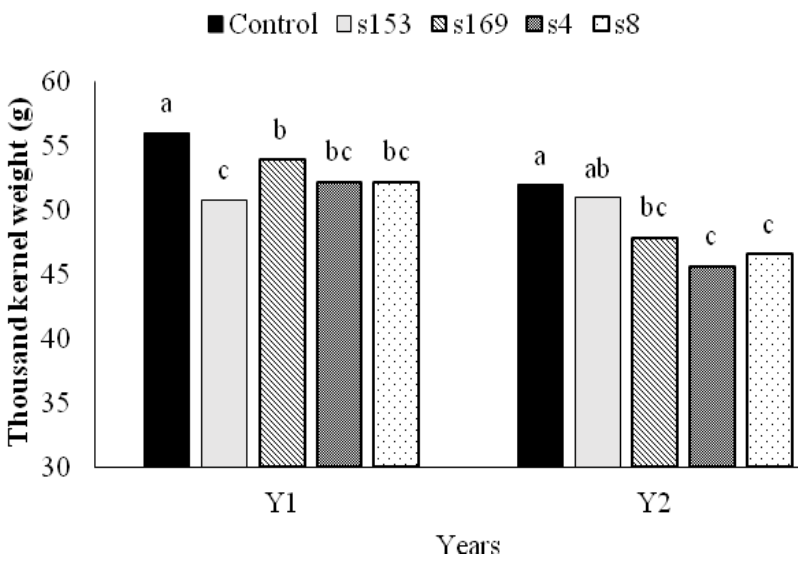

Figure 4. *Interaction effect of Pseudomonas strains × year on thousand kernel weight; Y1 = 2014-2015; Y2 = 2015-2016. Means in each column followed by at least one similar letter are not significantly different at 5% probability level, using LSD test*

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Biological yield

The interaction effect of *Pseudomonas* × AMF × cultivars × year on biological yield was significant ($P<0.05$) (*Table 3*). The comparison of mean showed that in the first year in the Dasht cultivar, the use of AMF increased the biological yield such that S4 strain (which showed the highest biological yield) in the conditions of AMF usage, caused increased biological yield of 22.73% in comparison to control. However, at the conditions of AMF non-use, biological yield increased by 14.66%. In the Sahra cultivar, S4 strain in both AMF using and non-using conditions had superiority in comparison to other strains of *Pseudomonas* (24.2% and 27.8% under AMF use and non-use conditions, respectively, in comparison to control), while significant difference was not found between some strains and control.

In the second year in the Dasht cultivar, the AMF use led to significant superiority of *Pseudomonas* strains compared to the control (10.2-18.3%), such that even in the control, the use of AMF increased biological yield by 5.47% in comparison to AMF non-use condition. In the Sahra cultivar in conditions of using AMF, only S4 strain had significant superiority compared to control (33.8%) and there was no significant difference between other strains of *Pseudomonas* and control (*Table 5*).

The increase in biological yield by soil and seed inoculation of *Pseudomonas* strains and AMF at different conditions has been reported by many researchers (Germida and Walley, 1996, Shaharuna et al., 2008). Khan et al. (2003) in the review of research conducted on growth stimulating bacteria reported the significant effect of inoculation with *P. fluorescent* on dry weight of aerial part of barley. Growth stimulating microorganisms such as *Pseudomonas* and AMF can provide optimum conditions to increase biomass by creating positive variations on soil physical and chemical properties around the root, and the timely provision of required elements during the growing season (Esfahani et al., 2009). Messele and Pant (2012) observed improved biological yield and P uptake in chickpea (*Cicer arietinum* L.) by phosphate-solubilizing *Pseudomonas*. An increase in P availability to plants through the action of phosphate-solubilizing bacteria (PSB) has also been reported for wheat (Panhwar et al., 2011). Application of plant growth stimulating bacteria that can also produce the enzyme ACC deaminase and alongside AMF can provide the elements needed by plant and lead to increased plant growth (Lingua et al., 2003). Their synergistic interaction increased N, P and K uptake by plants under water deficit conditions (Malfanov et al., 2011). Strains appear to have some plant growth-promoting activities, such as IAA production and solubilization of phosphate, which together or alone might explain the capacity of *Pseudomonas* strains to improve plant growth and nutrient acquisition.

Seed yield

The interaction effect of *Pseudomonas* × AMF × cultivars × year on seed yield was significant ($P<0.01$) (*Table 3*). A comparison of the mean interaction effect of *Pseudomonas* and the AMF on seed yield of barley cultivars revealed that in the first year at Dasht cultivar using AMF conditions, significant differences were not observed between *Pseudomonas* strains. However, the use of AMF led to increased seed yield in all strains and even in control and S4 strain with yield of 1293 kg/ha which increased seed yield by 17.63% compared to control. In the Sahra cultivar, *Pseudomonas* strains increased seed yield at both levels of AMF.

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In the second year at Dasht cultivar in both levels of AMF, strains of *Pseudomonas* when compared with control showed significant superiority and at the level of AMF non-use conditions, strain S8 with mean of 1529 kg/ha and at the AMF use conditions, strain S4 with mean of 1912 kg/ha (had no significant difference with S169) exhibited the highest seed yield. In the Sahra cultivar, *Pseudomonas* strains increased seed yield in comparison to control, and S4 increased seed yield by 13.33% at AMF non-use conditions and 18.66% at AMF use conditions (*Table 5*). The effect of microorganisms on plant depends on many factors such as soil organic matter, the amount of existing elements in the soil, soil texture, soil moisture, strain type, and plant cultivar (Vessey, 2003). The relationship between plant cultivars and strains have been reported in the studies of Kader et al. (2002) and Dijkman et al. (2017). Nonetheless, with increased seed yield in AMF non-use conditions, *Pseudomonas* strains compared to control caused increase in seed yield in Sahra cultivar, but the increase in seed yield is increased by adding AMF, and in the Dasht cultivar, its efficiency of bacterial inoculation is increased. The effect of AMF on the increased efficiency of bacterial inoculation has been reported by Sexena and Jha (2014). However, increase in seed yield can be attributed to the role of AMF and *P. Putida* in increasing uptake of elements such as Fe and P (Vessey, 2003). However, partial increase in barley seed yield can be attributed to the secretion of plant hormones secreted such as Auxin- by microorganisms. Secretion of IAA and other plant growth hormones in canola, wheat, and tomatoes inoculated with *P. Putida* has been reported by Mayak et al. (2004). In addition, plant growth stimulating microorganisms affect the root morphology and size and thus affect the root ability in accessing larger volume of soil. Consequently, they increase uptake of water and plants yield (Arshad et al., 2008).

Table 5. *Interaction effect of Pseudomonas strains × AMF × Barley cultivars on biological yield, seed yield and harvest index*

Barley cultivar	AMF	*Pseudomonas* strains	Biological yield (kg/ha)		Seed yield (kg/ha)		Harvest index (%)	
			Y1	Y2	Y1	Y2	Y1	Y2
Dasht	M0	Control	2269c	3453a	1007a	1141d	26.28b	33.20c
		S153	2781ab	3946a	1096a	1276c	26.72ab	39.42ab
		S169	2829ab	3893a	1106a	1377bc	27.44ab	38.35ab
		S4	2949a	4133a	1205a	1440ab	33.11a	40.50a
		S8	2493bc	3920a	1081a	1529a	28.55ab	35.72bc
Dasht	M1	Control	2439c	3653b	1065c	1198d	33.41a	42.46a
		S153	3120ab	4133ab	1212ab	1385c	29.88a	31.13b
		S169	2787c	4026ab	1179abc	1830a	33.51a	33.32b
		S4	3158a	4323a	1293a	1912a	26.52a	33.30b
		S8	2895bc	4090ab	1204ab	1642b	27.73a	33.41b
Sahra	M0	Control	2416b	2693b	1414c	1652c	37.28b	40.24a
		S153	2825ab	3293ab	1600b	1803ab	42.49ab	38.61ab
		S169	2935ab	3440ab	1564b	1771b	40.85ab	34.64b
		S4	3075a	3993a	1726a	1906a	45.40ab	34.82b
		S8	2638ab	3820a	1618b	1727bc	47.04a	37.92ab
Sahra	M1	Control	2524b	3466b	1447c	1577d	41.48ab	41.93a
		S153	2570b	3706b	1637ab	1837b	44.88a	39.72ab
		S169	2963ab	3560b	1465bc	1839b	40.46ab	37.46b
		S4	3150a	4640a	1752a	1939a	37.85b	40.08ab
		S8	2820ab	3506b	1491bc	1736c	38.83b	35.42c

Means within a column followed by the same letter are not significantly (P < 0.05) different according to LSD test. (Y1 = 2014-2015; Y2 = 2015-2016) (M0 = AMF non-use; M1 = AMF use)

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Harvest index

The interaction effect of *Pseudomonas* × AMF × cultivars × year on harvest index was significant ($P<0.01$) (*Table 3*). The mean comparison revealed that in the Dasht cultivar at the AMF use condition, there was no significant difference between strains, but at AMF non-use condition, *Pseudomonas* strains had significant superiority when compared to control and S4 showed the highest harvest index. In Sahra cultivar, at the AMF use conditions, strains S8, and at the AMF non-use conditions, S153 had the highest harvest index. In the second year, only the Dasht cultivar and at the AMF use conditions, strain S4 demonstrated maximum harvest index, but at the AMF use conditions in the Dasht cultivar and in Sahra cultivar at both AMF use and non-use conditions, control exhibited the greatest harvest index (*Table 5*).

N uptake and N concentration of seed

The interaction effect of bacteria × cultivar on seed N concentration was significant ($P<0.01$) (*Table 6*). A comparison of the mean interaction effect of bacteria × cultivar on seed N concentration showed that in the Dasht cultivar, only strain S8 had significant superiority compared to control (14.2%) and other strains demonstrated no significant superiority. Nevertheless, in the Sahra cultivar, strain S8 showed no significant superiority when compared to control, while other strains showed superiority when compared to control and the highest seed N concentration was obtained by S4 strain (28.7%) (*Figure 5*).

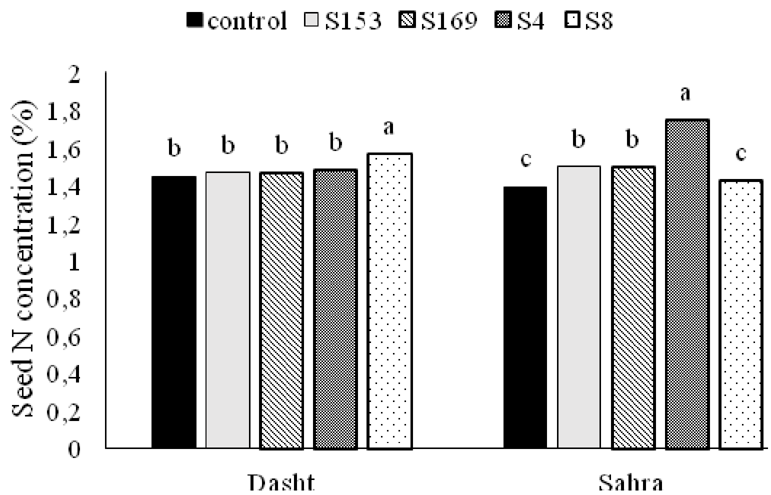

Figure 5. *Interaction effect of barley cultivars × Pseudomonas strains on seed N concentration. Means in each column followed by at least one similar letter are not significantly different at 5% probability level, using LSD test*

The results of the analysis of variance showed that the interaction effect of *Pseudomonas* × AMF × barley cultivars × year on seed N uptake was significant ($P<0.05$) (*Table 6*). A comparison of the mean interaction effect of *Pseudomonas* × AMF × barley cultivars × year on seed nitrogen uptake showed that in the first year in the Dasht cultivar at the AMF non-use conditions, strains of *Pseudomonas* exhibited significant superiority compared to control and S4 strain (20.19 kg/ha) demonstrated the

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highest nitrogen uptake, but in AMF use conditions, there was no significant difference between *Pseudomonas* strains. In the Sahra cultivar, in both AMF use and non-use conditions, strains of *Pseudomonas* exhibited significant superiority compared to control (8-49% and 2-40% under AMF use and non-use conditions, respectively, in comparison to control) and S4 strain (32.01 and 27.17 kg/ha under AMF use and non-use conditions, respectively) showed maximum nitrogen uptake (*Table 7*).

In the second year at Dasht cultivar in both AMF use and non-use conditions, strains of *Pseudomonas* exhibited significant superiority compared to control (22-65% and 16-69% under AMF use and non-use conditions, respectively, in comparison to control). In the Sahra cultivar, at the AMF non-use conditions, S4 strain (29.43 kg/ha) and in AMF use conditions, S8 strain (27.65 kg/ha) exhibited the maximum seed nitrogen uptake. In total, the highest nitrogen uptake in both years was seen in Sahra cultivar and in strain S4, while in the first year, it was obtained in the AMF use conditions and in the second year, it was found in AMF non-use conditions (*Table 7*).

Given that this experiment was conducted at rainfed and water shortage conditions, it could be stated that the growth period has been increasingly reduced and plant has been faced with fewer opportunities for the accumulation of starch in the seed. Consequently, seed protein content is increased. In addition, according to many researchers, through participation in the building of RNA polymerase, zinc caused increase in amino acids and the synthesis of proteins (Koutroubas et al., 2016). As a result, it increased protein content as suggested by the high content of zinc in inoculated treatments in this experiment.

The seed nitrogen uptake that depends on seed nitrogen concentration and seed yield was more in most of co-inoculated treatments of AMF + *Pseudomonas* compared to plants merely inoculated with AMF or *Pseudomonas*. It seems that in stress conditions, AMF played an important role in the formation and stability of soil aggregate. Consequently, its improved soil hydrolytic conductivity led to the development of root system and improved water and nutrients uptake (Estrada-Campuzano et al., 2012).

In evaluating the interaction between *Pseudomonas*, *Azotobacter* and AMF on uptake of elements in sorghum, Sultana et al. (2016) stated that AMF inoculation alongside *Pseudomonas* increased the concentration of iron, zinc, copper, and protein, but increase in manganese content was not significant. They believed that the increased uptake of iron, zinc, copper, and protein was due to impact of the seed co-inoculation *Pseudomonas* + AMF in increasing cell division and elongation in roots and thus lateral root development in co-inoculation treatments of *Pseudomonas* + AMF. However, Haling et al. (2010) studied the root of millets in food and water shortage conditions. They reported that in poor and alkaline soils, when the ability of root in the production of organic acids in the root environment is higher and guides the rhizosphere toward acidification, it could lead to uptake of elements such as N and P thereby leading to increased plant growth. Therefore, microorganisms used in this study have the potential to produce organic acids and they could increase uptake of nutrients by reducing the pH of the soil. However, Nogueira et al. (2007) assessed the impact of AMF and some PGPRs on nutrient uptake in soybean and reported the role of AMF in N uptake as result of indirect symbiosis of AMF and plant. They believed that AMF increased the uptake of elements such as P, Fe and Mn and it increased the uptake of nutrients by increasing the level of hyphae at the root surface, and this increase was independent from N uptake. In this experiment, the effect of inoculation in increasing the seed N in response to barley cultivar and year was different.

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P uptake and P concentration of seed

The results of the analysis of variance showed that the interaction effect of *Pseudomonas* × AMF × barley cultivars × year on P concentration was significant ($P<0.01$) (*Table 6*). By comparing the mean interaction effect of *Pseudomonas* × AMF × cultivars × year on P concentration, it was found that in both years and in both cultivars, *pseudomonas* strains were significantly superior to control. In both years, S4 strain in most treatment combinations of AMF × cultivar had significant superiority compared to other strains. The important point is the impact of AMF on increasing the concentrations of seed P so that in most *Pseudomonas* treatments, using AMF increased seed P concentrations significantly (*Table 7*).

Table 6. *Analysis of variance effect of barley cultivars under AMF and Pseudomonas inoculation on seed N concentration, N uptake, P concentration and P uptake*

S.O.V	df	Mean square			
		Seed N concentration (%)	N uptake (kg/ha)	Seed P concentration (%)	P uptake (kg/ha)
Y (Year)	1	0.0520ns	235**	0.142**	28.1**
Year / Rep	4	0.0142ns	94.2**	0.0014ns	0.086ns
Cultivar (C)	1	0.020ns	406**	0.069**	18.71**
AM fungi(M)	1	0.019ns	31.41*	0.109**	6.03**
Bacteria (B)	4	0.124**	104.1**	0.135**	10.86**
Y × C	1	0.001ns	3.236ns	0.0005ns	0.421*
Y × M	1	0.00007ns	6.25ns	0.006ns	0.000001ns
Y × B	4	0.051ns	21.81*	0.115**	0.070ns
C × M	1	0.0001ns	0.137ns	0.0003ns	0.847**
C × B	4	0.137**	15.19ns	0.004ns	0.510**
M × B	4	0.048ns	16.36ns	0.022*	0.757**
M × B × C	4	0.039ns	19.21*	0.0015ns	0.801**
Y× C × M	1	0.021ns	2.62ns	0.071**	0.986**
Y × C × B	4	0.059ns	35.33**	0.0015ns	0.080ns
Y × M × B	4	0.021ns	19.71*	0.0020ns	0.764**
Y × C × M × B	4	0.035ns	38.57*	0.133**	1.42**
Error	76	0.025	7.52	0.0037	0.082
CV (%)		10.68	12.1	17.5	5.41

**, ** Significant at $P < 0.05$ and $P < 0.01$, respectively. CV: Coefficient of Variation*

The results of the analysis of variance showed that the interaction effect of *Pseudomonas* × AMF × barley cultivars × year on P uptake by seed was significant ($P<0.01$) (*Table 6*). Comparing the interaction effect of *Pseudomonas* × AMF × cultivars × year on P uptake revealed that in both years and in each of the cultivars, *pseudomonas* treatments increased P uptake significantly in comparison to control. In the Dasht cultivar, in AMF use conditions, there was no significant difference between strains in the first year, but in the second year, this difference was significant and strain S8 showed the highest P uptake. AMF use in Dasht cultivar increased the difference between strains in both years so that in both cultivars, strain S8 had significant superiority when compared to other strains. In the Sahra cultivar, the process of phosphorus uptake was similar to Dasht cultivar, while in AMF non-use conditions in the second year, strain S4 showed the highest P uptake compared to other strains (*Table 7*).

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Table 7. *Interaction effect of Pseudomonas strains × AMF × Barley cultivars on biological yield, seed yield and harvest index*

Barley cultivar	AMF	*Pseudomonas* strains	N uptake (kg/ha)		Seed P concentration (%)		P uptake(kg/ha)	
			Y1	Y2	Y1	Y2	Y1	Y2
Dasht	M0	Control	17.22b	16.95d	0.283b	0.273d	2.83b	3.66d
		S153	18.79ab	19.78c	0.373a	0.326b	4.45a	4.82c
		S169	19.38ab	28.76a	0.350a	0.300c	4.37a	4.73c
		S4	20.19a	20.34c	0.363a	0.329b	4.55a	5.35b
		S8	18.22ab	23.73b	0.340a	0.366a	4.17a	6.34a
Dasht	M1	Control	18.87a	16.87c	0.306c	0.284d	3.40d	4.310e
		S153	19.92a	22.01b	0.350b	0.362bc	4.27c	5.746c
		S169	21.50a	26.66a	0.350b	0.372b	4.27c	6.772b
		S4	20.81a	27.95a	0.436a	0.393a	5.74a	7.137a
		S8	20.26a	20.73b	0.373b	0.336c	4.88b	4.940d
Sahra	M0	Control	19.35d	24.05b	0.286c	0.289c	3.98b	5.41c
		S153	24.07b	25.23b	0.383a	0.350ab	5.41a	6.22ab
		S169	19.79d	25.28b	0.350b	0.340b	5.03a	6.01bc
		S4	27.17a	29.43a	0.363ab	0.376a	5.28a	6.67a
		S8	21.64c	24.16b	0.343b	0.330bc	5.14a	5.69bc
Sahra	M1	Control	21.37c	21.34c	0.316b	0.327c	4.49c	4.83c
		S153	19.54d	26.39b	0.376a	0.369b	5.58b	6.36ab
		S169	24.40b	27.28ab	0.390a	0.354b	5.51b	6.19b
		S4	32.01a	26.51b	0.396a	0.403a	6.19a	6.65a
		S8	23.23b	27.65a	1491bc	0.358b	5.39b	6.46ab

Means within a column followed by the same letter are not significantly (P < 0.05) different according to LSD test. (*Y1 = 2014-2015; Y2 = 2015-2016*) (*M0 = AMF non-use; M1 = AMF use*)

Initial experiments on the strains of the bacterium used in this experiment show that *Pseudomonas* strains is able to dissolve the insoluble mineral P via the reduction of pH and it is able to produce and secrete acid and alkaline phosphatase enzymes and obtaining P from organic sources. Furthermore, efficiency of strain S4 in comparison with three other strains is higher in these characteristics. Therefore, it is obvious that the amount of P uptake and transport in strain S4 is higher and treatments inoculated had higher P content compared to control. Some believe that this increase in ionic content is related to the development of root systems and a general increase in the level of absorption of ions and is not associated with a specific mechanism (Guadarika et al., 2003). However, Kader et al. (2002) showed that bacterial inoculation affects ATPase and electrogenic pump in root cell membranes and by increasing leakage of protons from the root, it provides the driving force necessary to attract other ions for plant.

These microorganisms that have the ability to dissolve P mineral, and by secreting organic acids such as oxalic acid and citric acid, lead to the release of P into the soluble soil through chelating and the formation of stable complexes with iron, aluminum and calcium cations. In addition, 2-ketooxalic acid and glucuronic acid reduce the environment pH and soil insoluble phosphate solubilization by releasing protein (Salimpour et al., 2011). As a result of release of phosphorus in the soil, the root access to this element increases and the efficiency of P uptake in leaf increases. However, the type and amount of organic acids in any environment is related to the type of microorganisms producing the organic acid (Ghaderi et al., 2008). These results are in line with the results of the research conducted by Naseri et al. (2013). Saxena and Jha (2014) reported the greatest amount of P of wheat plant in relation to the co-inoculation of AMF + endophytic bacteria. The researchers reported the

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increased uptake of P by plants symbiosis with P solubilizing microorganisms due to the production of carbon by these microorganisms and its impact on increased ability in P uptake (Khan et al., 2013). Shaharoona et al. (2008) reported that *P. fluorescents* strain F increased absorption of N, P and K compared to non-inoculated plants while reforming the growth elements of the wheat.

The findings of this study showed that phosphate-solubilizing microorganisms could increase growth and phosphorus uptake in the atmosphere, leading to increased crop tolerance under water deficit stress. In this experiment, increased phosphorus in aerial organs led to an increased seed phosphorous content such that maximum phosphorous was observed in the same strain. Therefore, we can say that any factor that increases phosphorous of aerial organs transports more phosphorus to seed, leading to an increased level of phosphorous content.

Conclusion

Given that this experiment was conducted in rainfed conditions, rainfall is the factor of change in seed yield and other physiological and biochemical reactions. In this experiment conducted in two consecutive years under rainfed conditions in Ardebil on barley cultivars under interaction between AMF and *Pseudomonas* strains, seed yield in both cultivars in the second year was higher due to more rainfall and effective dispersion (in terms of growth to plant barley). For this reason, reaction of cultivars inoculated treatments compared to first year was different. In both cultivars, maximum seed yield in both years was obtained from co-inoculation treatment of AMF + strain S4, while Sahra cultivar had more production sustainability compared to Dasht cultivar. While maximum seed yield in both cultivars was obtained from the same treatment, Sahra cultivar exhibited the better reaction to inoculation. The high seed yield in Sahra cultivar compared to Dasht cultivar can be attributed to reasons such as increased number of fertile tillers and number of seed per spike. Increase in the number of seed per spike and increase in fertile tiller can be due to proper nutrition and providing nutrients by AMF and *Pseudomonas*, since co-inoculation increased concentration of N and P significantly. However, the capability of AMF fungi in increasing uptake level that increases the uptake and retention of water in the rhizosphere plant should not be ignored. In addition, strains used in this experiment were associated with the production of the enzyme ACC deaminase and reduced levels of ethylene production (in rainfed conditions in which the plant is faced with water shortage and ethylene produced in the incidence of stress). Although in most of the characteristics measured, inoculated treatments were superior compared to control, the use of AMF also increased bacterial inoculation efficiency and by increasing the uptake of nutrients and increasing tolerance to water shortage conditions, it increased the seed yield, while this increase was more evident in the Sahra cultivar. Therefore, to plant barley in the rainfed conditions of Iran, Sahra cultivar alongside co-inoculation AMF and *P. putida* strain S4 is recommended. The development of drought-tolerant crop varieties through genetic engineering and plant breeding is essential but it is a long drawn process, whereas PGPR inoculation to alleviate drought stresses in plants opens a new chapter in the application of microorganisms in dry land agriculture. Taking the current leads available, concerted future research is needed in terms of identification of the right kind of microbes and barley cultivars and addressing the issue of delivery systems and field evaluation of potential organisms at diverse agro-ecological conditions under N and P fertilization.

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ESTIMATION OF EVAPOTRANSPIRATION BASED ON SURFACE ENERGY BALANCE ALGORITHM FOR LAND (SEBAL) USING LANDSAT 8 AND MODIS IMAGES

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Abstract. The aim of this study was to determine the accuracy estimation of actual evapotranspiration by using Surface Energy Balance Algorithm for Land (SEBAL) in comparison with FAO-Penman-Monteith method in Landsat and MODIS images in Malayer County of Iran. In this study, Landsat 8 and MODIS images of 2013 were used. Result showed that there are not many differences between SEBAL and FAO-Penman-Monteith methods in estimation of evapotranspiration. According to the aim of the study, result showed that MODIS sensor with high accuracy have calculated evapotranspiration more accurately than Landsat 8 by RMSE = 1.004 and MBE = 0.0033 respectively in surface temperature estimation which has great effect on estimation of the net radiation flux, soil heat flux and the sensible heat flux. On the other hand, according to low spatial resolution of MODIS sensor, we can conclude that this sensor has less value for zoning of evapotranspiration than Landsat 8 image according to the area topography and heterogeneous land uses.
Keywords: *evapotranspiration, remote sensing, SEBAL, Malayer city, MODIS, Landsat 8*

Introduction

Evapotranspiration (ET) is an important component of water balance in arid and semi-arid areas and accurate estimation of ET is significantly important for optimum management of water resources (Sepaskhah, 1982). Hasheminia (1999) reported, 70 % of precipitation returns to the atmosphere through ET. He also cited in arid areas it is about 90 %. According to his output, water shortage problem has been widespread in many parts of the world which has brought about conflict over water in the near future. It seems that limitation of water resources and uneconomical uses of water are the main factors that limit the development of agriculture and increase food production in Iran (Akbari, 2004). On the other hand, ET plays significant role in the world's climate through hydrologic cycle. ET estimation has an important utilization on runoff prediction, product performance prediction, land use design, design of irrigation channels, water distribution constructions and it is also effective on natural disasters like drought (Ogawa et al., 1999; Bastiaanssen, 2000; Norman et al., 1995). Many methods have been developed for calculating ET in different geographical and climatic conditions by using meteorological data. These methods often use point (data) measurement for ET estimation, so they are appropriate for local area and they are not expendable to large basins because of dynamic nature and regional changes of

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ET (Li and Lyons, 1999; 2002). Development of remote sensing technology provides possibility of actual ET and potential ET estimation in a wide scale. In many researches satellite images were used to estimate actual ET and temporal and spatial distribution. Remote sensing is able to estimate ET also it can examine spatial distribution, because it can extract parameters such as surface temperature, albedo coefficient and vegetation index which is compatible with the environment and it is economically affordable (Norman et al., 1995). This distribution is important for management at the macro level. In the irrigation conference which was held by Food and Agriculture Organization (FAO) and International Commission on Irrigation and Drainage (ICID) and World Meteorological Organization (WMO) in cooperation in 1990 for investigating FAO methods and presenting an accurate method, FAO-Penman-Monteith method proposed as a standard method for ET estimation (Allen et al., 1998). ICID and FAO proposed FAO-Penman-Monteith method as a standard method for ET calculation via climate data (Hargreaves, 1994). Heretofore, the multiple algorithms have been provided to estimate ET using remote sensing data. Thereby, the methods based on energy balance are divided into single-source and two-source models. Single-source models consider soil and plant as one source named “Big-Leaf” and they only use an aerodynamic resistance in water-heat transition process (Nishida et al., 2003). In these models, it is assumed that the entire affected surface receives the same temperature and humidity. Unlike single-source models, two-source models by separation of soil and plants in all modeling process use several distinct aerodynamic resistances for soil and plant (Huntingford et al., 2000). Among the popular models in single-source base, we can mention Surface Energy Balance for Land (SEBAL), Mapping Evapotranspiration at High Resolution with Internalized Calibration (METRIC) and Surface Energy Balance System (SEBS) (Bastiaanssen et al., 1998; Su, 2002; Allen et al., 2007; Ogawa et al., 1999). The models such as TSEB and STSEB are two-source models (Norman et al., 1995; Sánchez et al., 2008).

Review of literature

The SEBAL algorithm was used with different sensor images in multiple parts of the world and it provided acceptable results. The old SEBAL model with METRIC in Idaho and their output results were compared which had a good agreement with lysimeter data (Tasumi et al., 2005). In another study daily ET amounts with lysimeter data by using Landsat images and METRIC model were compared and error less than 28 % was reported (Chavez et al., 2007). Estimation of ET by using Landsat images and energy balance METRIC model in Brazil had highlighted the model performance of water consumption estimation and improved water management in semi-arid and irrigated area in northeast of Brazil (Folhes et al., 2009). Evapotranspiration by using SEBAL algorithm and water balance model was calculated and results were compared, correlation by 70 % was reported (Mutiga et al., 2010). Useful results were reported about using Landsat images and SEBAL algorithm for actual ET estimation in multiple land uses of Nansi Lake basin, China (Sun et al., 2011). Surface temperature, net radiation, soil heat flux and hourly ET amounts were calculated by using SEBAL algorithm and 16 TM images were used in Texas plain. Output results were compared with measured values of 4 lysimeters which in cotton planted in two modes: irrigated and under water stress. Results

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showed high accuracy of output results (Colaizzi et al., 2011). Different methods of ET estimation based on remote sensing data were investigated and results indicated that remote sensing methods have average accuracy for ET estimation (Raghuveer et al., 2011). Variable ET on the agriculture land, water body, forests and grasslands, are justifiable with different amount of ET (Yuting et al., 2012). Measured ET and other SEBAL components with measured field data through 4 accurate weighty lysimeters in two areas wet and dry farming were compared and acceptable results by using SEBAL were emphasized (Paul et al., 2013). Actual ET of pistachio in Ardakan, Yazd, by using 12 MODIS images and SEBAL algorithm were calculated. Results showed that average actual ET of pistachio is 1123 mm in desired year during a complete grow season which is much less than the amount of water consumption (Dasturani et al., 2012). Estimation and comparison of ET by using SEBAL algorithm, MODIS and Landsat 7 images were done and it was concluded that TM sensor image accuracy was about two and a half times more than MODIS (Simaie et al., 2013).

Although a huge number of studies have used different algorithms including SEBAL and multiple satellite images such as MODIS and Landsat. Over two decades studies were done about this issue, it is necessary to study different climate and weather for an accurate evaluation of ET based on these models and satellite images. Whereas, these models are experimental and they use a set of methods and experimental equations, they have to be calibrated for regional conditions. In the present study, actual ET have been estimated in Malayer city which has heterogeneous land uses such as range, residential, agricultural land (wet and dry), water bodies and so on. It should be mentioned that there is no record of any study using MODIS, Landsat or even other remote sensing images such as SEBAL or other models about ET estimation in the study area. However, most previous studies considered ET estimation in agricultural areas, either in an area with different land uses or new Landsat 8 satellite image, but the present study with an emphasis on performance of SEBAL, MODIS and Landsat 8 sensors have been investigated in a heterogeneous area.

Material and methods

Malayer city in Iran has an area of 3208 km^2 which includes 16.9 % of Hamadan province (*Fig. 1*). Elevation changes between 1617 and 3345 m (*Fig. 1,* digital elevation model is shown). The average amount of annual precipitation ranges is between 250 and 327 mm based on elevation gradient and extracted precipitation from whole city stations and neighborhood stations (Nahavand, Toiserkan, Hamadan Airport, Nouzhe, Arak, Kangavar and Borojerd) in a 17 years period (1995 to 2012). The amount of 24 hourly potential ET has been recorded by 7.58 mm averagely in Malayer station in May. Lysimeter data is usually used in different remote sensing studies for ET estimation. According to lack of lysimeter station in Malayer, synoptic station which records temperature, precipitation and evaporation are used as observed station to control output results (*Fig. 1*). Hourly data of 13 May 2012 at the same time with time recording of MODIS and Landsat 8 images were used by FAO-Penman-Montieth. Since the amount of precipitation and agriculture lands have been affected by water scarcity, thereby water consumption can be managed through ET estimation to reach a high efficiency.

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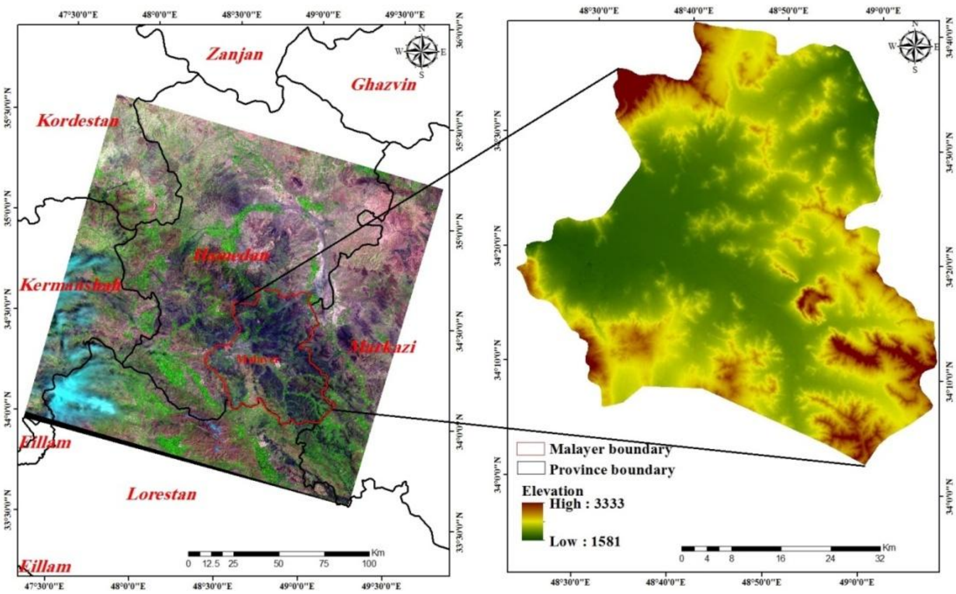

Figure 1. *Geographical location of study area in Hamadan province*

Image selection, preparation and preprocessing

One of the suitable images that are used around the world is free MODIS image. New free Landsat 8 images (OLI sensor) are one of the new feature in this case. Another point that should be mentioned is calibration of different images especially cheap images to local condition. Although MODIS image had been used in around of the world and also Iran, but these images and different algorithm including SEBAL are in the stage of development. Furthermore, significant research does not fulfillment in the field of use or calibration of different images including MODIS and Landsat 8 (OLI) images in Hamadan province which has distinct vegetation and climatic condition. In the present study images which have been selected are MODIS and Landsat 8 images for comparing factors including temporal condition (spring), desirable quality and lack of cloud cover spots. Although, the selected images were Level-II and they do not need geometric correction, but they were controlled with 10 ground control point (GCP) of GPS before using. Images did not need atmospheric correction because there was no atmospheric turbulence and they were one-temporal interpretation (Song et al., 2001). *Table 1* presents used images properties.

Table 1. *Properties of selected OLI image (Official Landsat 8 Site, 2013) and MODIS image*

MODIS	Landsat OLI	Image
05/14/2013	05/14/2013	Data catch
64-95	125.471-128.2050	Sun azimuth angle
36 (9 reflective, 17 thermal)	11 (9 reflective, 2 thermal)	Number band
250, 500 & 1000	15 & 30	Spatial resolution

Surface energy balance on land (SEBAL) algorithm

Theoretical basics of ET calculation using SEBAL method are provided in different references detailed. In this model, actual ET is calculated by using satellite images

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based on balance energy equation. Selected satellite images can only provide information about past time, so mentioned models are allowed possibility of estimating instantaneous latent heat flux in time imagery (Allen et al., 2007). Latent heat flux was calculated by using *Equation 1* for each pixel of image.

$$\lambda ET = R_n - H - G \quad \text{(Eq. 1)}$$

where λET is latent heat flux (W/m^2), R_n is net radiation flux on land surface (W/m^2), G is soil heat flux (W/m^2) and H is sensible heat flux (W/m^2). SEBAL method was presented first time in order to estimate ET in agricultural and plain areas. But it is corrected in new version (Allen et al., 2007) which is presented for application in rugged and mountainous areas. In the present study new version of SEBAL were used because of Malayer condition which has heterogeneous land use. Accuracy of latent heat flux estimation depends on calculation process and accuracy of H, G and Rn estimation. The value of net radiation that is the first calculation to solve surface energy balance equation was calculated using *Equation 2* and incoming and outgoing radiation fluxes (Allen et al., 2007).

$$R_n = (1-\alpha)R_{S\downarrow} + R_{L\downarrow} - R_{L\uparrow} - (1-\varepsilon_0)R_{L\downarrow} \quad \text{(Eq. 2)}$$

where $R_{S\downarrow}$ is incoming short wave radiation (W/m^2), $R_{L\downarrow}$ is incoming long wave radiation (w/m^2), $R_{L\uparrow}$ is outgoing long wave radiation (W/m^2), α is surface albedo and ε_0 is surface radiation power of 31^{th} and 32^{th} MODIS bands and 10^{th} and 11^{th} Landsat 8 bands. Soil heat flux (G) is the amount of heat transfer in soil and vegetation via molecular conduction. Since it is difficult to calculate the amount of soil heat flux using satellite image directly, so first G/R_n ratio calculated in middle of the day using experimental equation (Bastiaanssen et al., 1998) (*Eq. 3*).

$$\frac{G}{R_n} = \frac{T_s}{\alpha}(0.0038\alpha + 0.0074\alpha^2)(1 - 0.98NDVI^4) \quad \text{(Eq. 3)}$$

where T_s is surface temperature (°C) and α is surface albedo. The G value was obtained by multiplying the above ratio in Rn. If NDVI value is less than 0, then the area considered as water and G/R_n ratio considered equal to 0.5. The area having T_s less than 4 °C and α more than 0.45 considered as snowy area and G/R_n ratio was applied equal to 0.5. Sensible heat flux (H) (the loss of heat to the air via molecular convection and conduction) is because of temperature difference which calculated using *Equation 4* (Allen et al., 2007).

$$H = \frac{\rho . Cp . dT}{r_{ah}} \quad \text{(Eq. 4)}$$

where ρ is the air density (kg/m^3), C_p is the specific heat of air (1004 J/Kg/K), dT is temperature difference (°K) between two elevation (Z_1-Z_2) and r_{ah} is aerodynamic resistance against heat transmission (s/m). Sensible heat flux is a function of the temperature gradient, surface roughness and wind speed. Solving the above equation is difficult because of existence of two unknown parameters including r_{ah} and dT and therefore we used two cold and hot pixels of the study area (which reliable values are predictable for H and so present estimation of dT) and wind speed in the certain height.

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Regarding that r_{ah} was a function of sensible heat flux, *Equation 4* did not have explicit solution and it was solved based on cyclic method. In order to correct atmospheric stability Monin-Obukhov length was used. The cold pixel selected from an area covered with complete vegetation and irrigated completely based on mentioned instructions which it is assumed surface temperature equal to near surface temperature. As the same way, the hot pixel selected from an arid area without any vegetation which it is assumed ET is 0. In selection of these pixels, some factors such as surface temperature, albedo and vegetation indices were used. That the cold pixel had low temperature, albedo of about 0.22 to 0.24 in accordance with alfalfa land and high NDVI, while hot pixel had high temperature, high albedo similar to other dry lands and without vegetation and low NDVI. In selection of hot/cold pixels, it considered to avoid very low or very high temperature selection. After correction of the sensible heat flux amount based on atmospheric condition, the instantaneous amount of latent heat flux calculated for each pixel of both images using *Equation 1*. Since the outcome amounts of net radiation flux (R_n), sensible heat flux (H) and soil heat flux (G) were instantaneous amounts and for satellite pass time, the amount of latent heat flux (λET) calculated instantaneous too. Therefore, ET amount was obtained from numerical calculation of λ and by dividing the number of per-pixel. The amount of ET was obtained using instantaneous latent heat flux and *Equation 5*.

Latent heat flux and instantaneous ET

Latent heat flux is the amount of heat loss from surface due to ET process which was obtained using *Equation 3*. Since the amounts of net radiation flux (R_n), sensible heat flux (H) and soil heat flux (G) are instantaneous amounts then the amount of latent heat flux (λET) is momentary too. λET is the amount obtaining from satellite image. Therefore, we should calculate λ numerical amount so the amount of ET is obtained by dividing the number of per-pixel.

The amount of ET is obtained using instantaneous latent heat flux as:

$$ET_{inst} = 3600\frac{\lambda ET}{\lambda} \qquad \text{(Eq. 5)}$$

where ET_{inst} is the amount of instantaneous ET (mm/hr), λ is evaporation latenet heat (J/Kg) and 3600 number convert the time from seconds to hours. The amount of λ is obtained using *Equation 6*.

$$\lambda = [2.501 - 0.00236(T_S - 273.15)] \times 10^6 \qquad \text{(Eq. 6)}$$

Ratio of reference ET

Ratio of reference ET is defined as ratio of instantaneous ET (ETinst) of each pixel (mm/hr) to reference ET (ETr) obtained from meteorological data for image time (mm/hr) (*Eq. 7*).

$$ET_rF = \frac{ET_{inst}}{ET_r} \qquad \text{(Eq. 7)}$$

ET_rF is similar to vegetation coefficient (Kc) and used to extrapolate of ET from image time to 24 h period or much longer. The amount of ET_rF usually ranges between

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0 and 1, so in a completely dry ET = 0 and $ET_rF = 0$, but in case of the cold pixel in an alfalfa or corn, ET is a few more than ET_r and so $ET_rF > 1$ (1.1 likely). The negative amount for ET_rF is because of systematic errors which enter to SEBAL via various assumptions.

Various equations are developed to calculate ET_r, out of which, Penman-Montieth equation is proposed by FAO to most countries, such as Iran, having arid and semiarid climate. Penman-Montieth method has various versions; many of the researchers use FAO-Penman-Montieth version as one of the most reliable method for ET estimation (Alizade, 2002). Therefore, FAO-Penman-Montieth version 56 was used in REF-ET software for ET_r calculation.

24 hourly ET

The daily amount of ET (ET_{24}) usually has more application than the instantaneous amount. SEBAL calculate ET_{24} amount assuming instantaneous ET_rF is similar to average ET_rF during 24 h. The amount of ET_rF (mm/day) is calculated as (*Eq. 8;* Allen et al., 2007):

$$ET_{24} = ET_rF \times ET_{r\_24} \quad \text{(Eq. 8)}$$

where ET_{r_24} is sum of ET_r during 24 h for the same day image recorded which is obtained by adding up the values of the hourly ET_r together on the satellite pass day.

Accuracy assessment

Hourly and 24 h values of ETr obtained via FAO-Penman-Montieth method using hourly data of Malayer meteorological station was used to validate SEBAL model. Root Means Square Error and Mean Bias Error were used for statistical assessment of the models accuracy. The value of RMSE presents the amount of model error (Dashtaki et al., 2010). MBE index shows trend to overestimation or underestimation. Mathematical descriptions of these statistics are as follows (*Eqs. 9* and *10*):

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(Yest,i - Yobs,i)^2} \quad \text{(Eq. 9)}$$

$$MBE = \frac{1}{n}\sum_{i=1}^{n}(Yest,i - Yobs,i) \quad \text{(Eq. 10)}$$

where Yest is the projected amount, Yobs is the measurement amount and n is the number of data.

Results

The maps of surface temperature distribution, vegetation index and daily ET are shown in *Figure 2*. Surface temperature and vegetation index are two important incomes to SEBAL model. These two indices, which are also applied to select hot/cold pixels, have a strong inverse correlation in case of accessing sufficient nutrient (Ruhoff et al., 2012). As shown in *Figure 2*, daily ET distribution shows a wide range of values regarding heterogeneous lands. Also, results show that an area which has high

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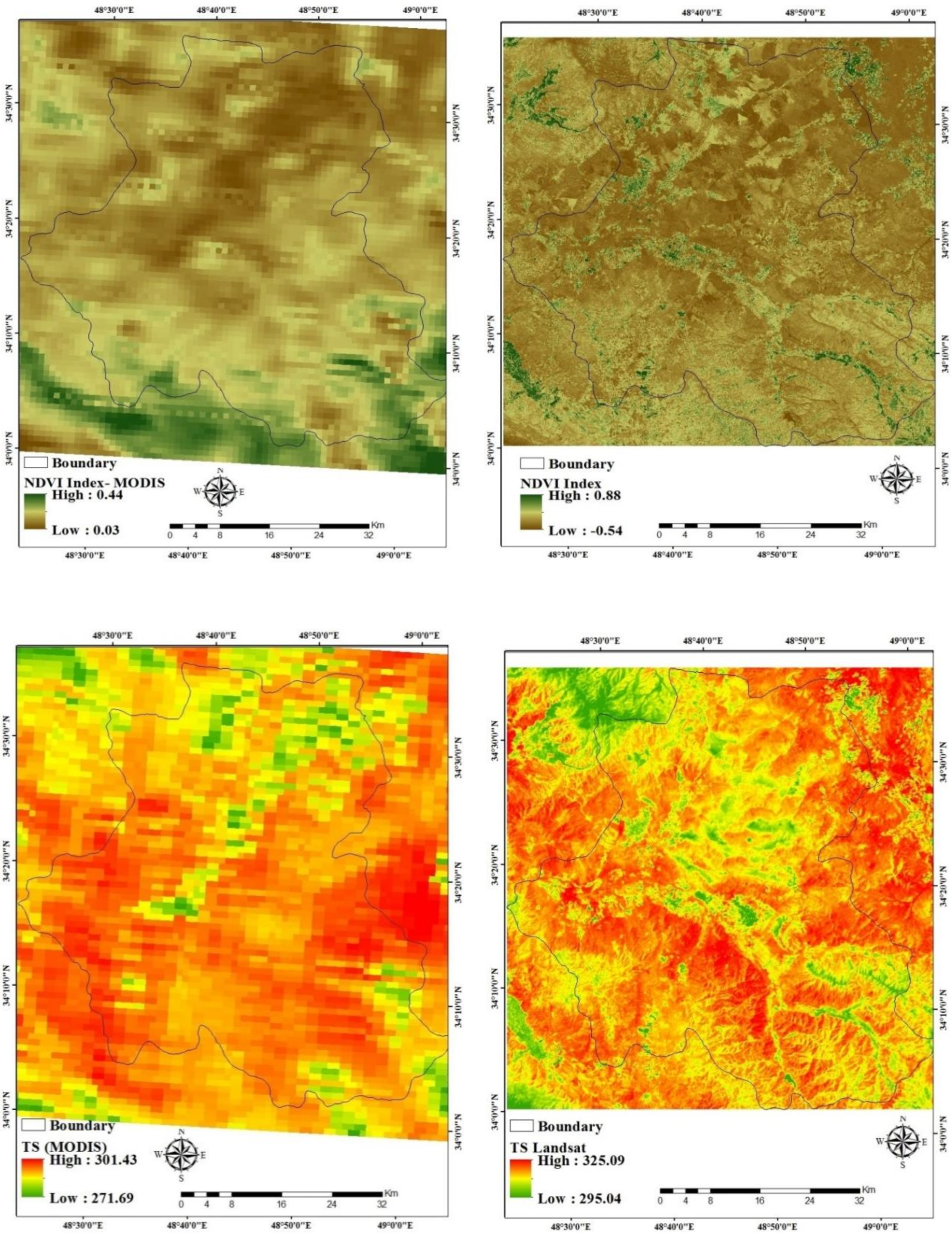
vegetation index (VI) and low temperature has more ET and vice versa. Based on the maps, there is an inverse correlation between surface temperature distribution and vegetation index.



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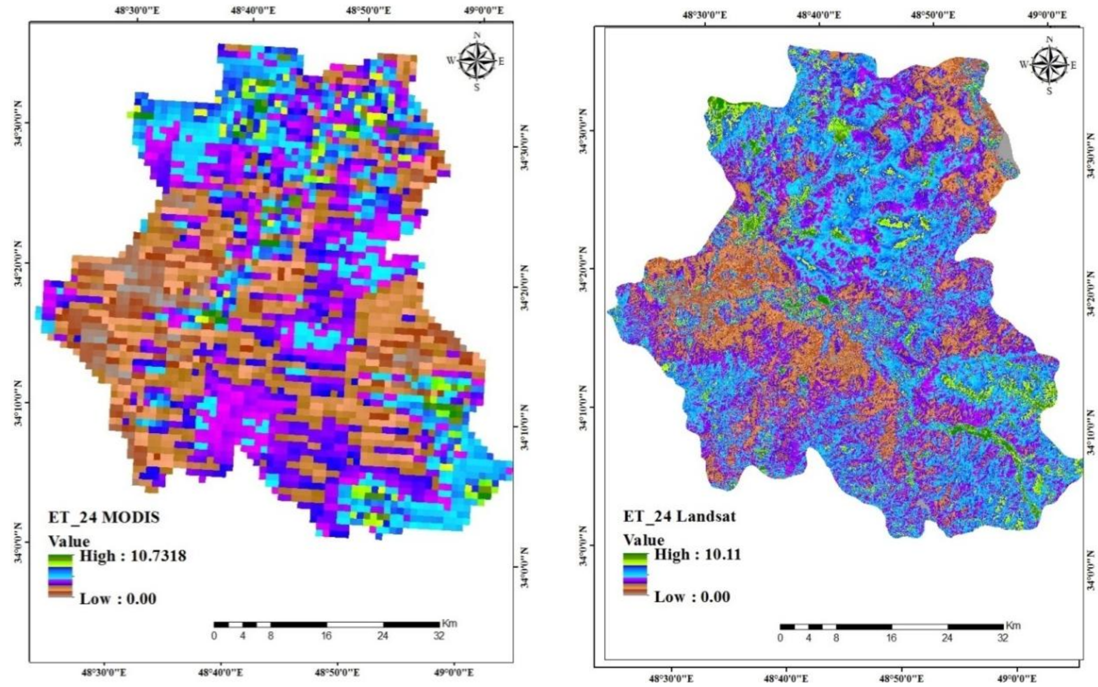

Figure 2. *Top to bottom and left to right are: MODIS surface temperature, Landsat surface temperature, NDVI of MODIS, NDVI of Landsat, 24 hourly ET of MODIS, 24 hourly ET of Landsat respectively*

Actual ET estimation

After calculating the net radiation flux (Rn), sensible radiation flux (H), soil heat flux (G) and latent heat flux of instantaneous evaporation, the amount of actual instantaneous ET (ETinst) was estimated.

The crop type and culture date were determined in desirable parts by using agriculture calendar. Then, the observed ET value of cultivated plants was estimated by using FAO-Penman-Montieth method and compared with results of SEBAL. The results of statistical comparison of SEBAL and FAO-Penman-Montieth are presented in *Table 2* and *Figure 3*.

Table 2. *Statistical comparison of SEBAL with FAO-Penman-Montieth (daily and hourly)*

Image	ET instant	ET ref instant	ET 24	ET ref 24
MODIS	0.7533	0.75	6.89	6.86
Landsat	0.7888	0.75	7.23	6.86

The ET amount measured by Landsat showed more differences with observed values. According to hourly ET measured through ETr software and calculated instantaneous values via satellite images showed MODIS accuracy is higher than Landsat with RMSE and MBE by 1.004 and 0.0033 respectively in comparison with Landsat with RMSE and ME by 1.05 and 2.88, respectively. But regarding images type and spatial resolution, it can be seen that accuracy of Landsat is much higher than MODIS image and it can interpolate the values of ET much better and clearer because

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of its smaller pixels. Ogawa et al. (1999) investigated performance of MODIS sensor in ET estimation in Savanah, West Africa. In that study, SEBAL method, MODIS and ASTER images were used. Their results showed both sensors have good capability to estimate ET in large heterogeneous areas and according to their results the present study has logical results in comparison with observed method. Sun et al. (2011) used SEBAL algorithm and Landsat image to estimate actual ET in various land uses in Nansi Lake Basin, China. These researchers reported positive results, similar to the present study which has emphasized on SEBAL capability in Malayer city.

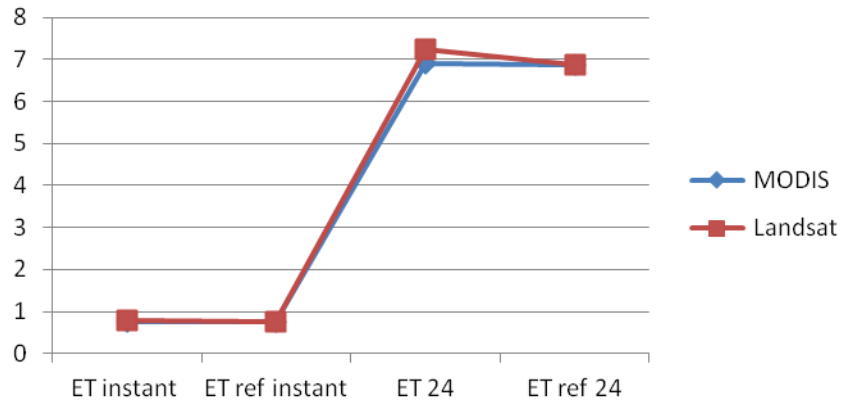

Figure 3. *Plotting comparison of SEBAL with observed method (FAO-Penamn-Montieth)*

According to assumption of SEBAL model based on *Equation 8*, daily ET was estimated. On the other hand, after correction of Kc based on proposed in FAO 56 leaflet for the study area and multiplied by observed ET of alfalfa which is obtained from meteorological data, daily actual ET values were estimated for desirable pixels. As shown in *Table 2*.

Conclusion

The greatest difference between SEBAL estimation and observed method is related to Landsat image. Based on daily ET obtained from ETr software, daily values that are calculated through satellite images and their statistical comparison, it is concluded that MODIS is more accurate than Landsat image and has less difference to observed ET. However, according to *Figure 2* which shows daily ET and high spatial resolution of Landsat, precision of this image is much higher than MODIS image and it can show ET clearer and more suitably. According to SEBAL estimations despite the error which caused deviation and due to lack of needed equipment and image errors, it can be said that data obtained from this algorithm are acceptable.

The aim of this study was to estimate ET of vegetation by MODIS and Landsat 8 images. For this purpose, SEBAL algorithm was used. Besides, Malayer city does not have any lysimeter station so FAO-Penman-Montieth’s results were used for accuracy assessment. Results showed SEBAL algorithm can estimate parameters such as surface albedo, surface temperature and vegetation index as well. Daily and hourly actual ET values were estimated in the study area by using mentioned parameters. Comparison of results from SEBAL algorithm and observed method (FAO-Penman-Montieth) did not show significant difference in either hourly or daily cases. It means hourly ET can be

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estimated with acceptable precision in comparison with observed ET in the study area. According to spatial resolution and free MODIS and Landsat images, it seems that the use of these images can be satisfactory and practical in order to estimate daily water requirement and plan for irrigation especially in wide lands.

The results of this study showed hourly estimations of ET through SEBAL algorithm have higher precision than daily estimations that is because of constant hourly ETref with daily ETref in model. Furthermore, comparison of estimating ET by two images which have different resolution made it clear that ET in MODIS image was estimated with higher accuracy for two anchor pixels (hot and cold pixels) than Landsat image. Nevertheless, according to observed ET, Landsat images had more accuracy due to higher spatial resolution and various heterogeneous land uses with low extent of study area.

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NUTRIENT VARIABILITY IN MANGROVE SOIL: ANTHROPOGENIC, SEASONAL AND DEPTH VARIATION FACTORS

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Abstract. The aim of this study is to assess the soil texture and nutrient components across three seasons at three different mangroves habitat according to the land used types; Kelantan (human settlement area); Johor (protected Ramsar's site) and Selangor (agricultural). The soils were sampled one-meter deep and divided into five segments for analysis. The pH of surface water for Selangor and Kelantan were slightly acidic ranging from 6.4 to 6.9. In Johor, the water was basic with value ranging from 8.3 to 8.7 across all seasons. Salinity in Selangor (26 ppm) was higher than in Johor (16 ppm) and in Kelantan (11 ppm). Johor and Selangor soils were dominated by silty loam, while Kelantan was dominated by sandy loam. Carbon, nitrogen, phosphorus and potassium analysis showed that they were significantly different in all locations but not between seasons. Except for phosphorus in Johor, consecutive depth did not influence the nutrient availability in mangrove soil. The results for carbon and nitrogen were following this order; Johor > Kelantan > Selangor, phosphorus; Selangor > Johor > Kelantan, potassium; Johor>Selangor>Kelantan. The protected mangroves habitat in Johor has siltier and clayey component and can retain more nutrients for the plant growth. Therefore, the conservation and preservation of mangroves habitat is crucial for the stable coastal ecosystems.
Keywords: *soil texture, carbon, nitrogen, phosphorus, potassium*

Introduction

Mangroves are a diverse group of predominantly trees, shrubs, palms and grounds ferns, which have adapted to the extreme saline conditions between the tides (Duke et al., 2002). They are one of the most productive and unique ecosystems, growing on sheltered shores and in estuaries in the tropical and subtropical area (Hogarth, 2015). Although the number of species diversity is low as compared to terrestrial ecosystems, it is the various adaptation abilities to survive the harsh environment (e.g. strong wind, high salinity and muddy substrate) that makes this ecosystem so critical and crucial for conservation. The largest mangroves areas are located in Asia, extending over 6.8 million ha and representing about 34-42 % of the world's total (Giri et al., 2011). Furthermore, many mangroves species were located at the equatorial area or country such as Indonesia, Malaysia and Philippines (Spalding et al., 2010). This forest is high in values, serves multiple ecological roles and important for socio-economic and microbes continuity (Walters et al., 2008). For instance, the high demand of charcoal and firewood had resulted in high production of mangrove woods in southern region of

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ASEAN, estimated around 10 to 17 tonnes/ha/year (Bosire et al., 2008). In Peninsular Malaysia, fish landing alone has been reported as much as 0.86 million tonnes in 2003 and this number increased every year (Chong, 2007).

Therefore, over the past decades, mangrove ecosystem became a subject to a high pressure of anthropogenic activities and natural phenomenon (Giri et al., 2011; Jusoff, 2013). Reclamation of mangroves area for development purposes; i.e. industrial area, human settlement, port and agriculture has been increasing and such acts have caused irreversible damage in coastal area. Erosion and accretion of the coastline regions are changing the sediment properties, physical and/or chemical ones (Kamaruzzaman et al., 2008). The mangrove forest clearance activities through unsustainability logging decreased the nutrients concentration in soil (Ngole-Jeme et al., 2016). This is due to the lack of litter production in mangroves ecosystems (Hemati et al., 2015). In addition, the climate change has contributed to sea level rise which directly causes the coastline changes and alter the mangrove coverage worldwide. This will change the coastline landscape and mangroves area that could change the soil physical and chemical properties (Tajul Baharuddin et al., 2013).

A good establishment of mangrove stands relies on the soil properties and this has been reported by several authors (e.g. Li et al., 2008; Kamali and Hashim, 2011; Salmo et al., 2013). Soil provides a good source of nutrient for growth and strong physical structure for anchorage and stability being in the soft sediments (Ashman and Puri, 2002). In mangroves, the sediment or soil texture, salinity and pH are important physical elements in determining the condition of the study area. The ability of soils to retain C, water and nutrient ions are strongly influenced by the soil texture (Havlin et al., 2014). Basically, soil texture is determined through the particle size percentage of clay, silt and sand (Ashman and Puri, 2002). The mixture of the results in actual mangrove sedimentation is classified as sand, loamy sand, sandy loam, loam, silt loam, silt, silty clay, silty clay loam, clay loam, sandy clay loam, sandy clay and clay. Most of the mangrove soils are known as mud, which is the mixture of silt and clay (Spalding et al., 2010).

The study on soil texture is important as mangrove plants may grow in different types of soil, hence determining the mangroves zonation pattern (Vilarrubia, 2000; Sherman et al., 2003; Twilley and Day, 2012). The soil pores are different for clay, silt and sand. For clay, the soil pores are the smallest when compared to silt and sand. Hence, this type of soil has a higher ability to retain water and nutrients (Ashman and Puri, 2002). Sasternegara (2004) reported that the silty clay is mostly found at the mangroves area, either logged or undisturbed mangroves area. But some species may favor other types of soil. *Avicennia* sp. can be found at the seaward zone with sandy soil type (Lewis, 2005; Ibrahim and Hussain, 2012). While for other species like *Rhizophora* spp. and *Bruguiera* spp. are mostly found in silty clay area (Robertson and Alongi, 1992; Ibrahim and Hussain, 2012).

Apart from soil texture, pH and salinity also play an important role in mangrove distribution. Several studies of mangrove forests worldwide had found that mangrove soil may be either acidic or alkaline (Wakushima et al., 1994; Salmo et al., 2014; Hemati et al., 2015). In the Sibuti Wildlife Santuary Miri, Sarawak, the pH was acidic with average 3.34 in the area dominated with *Rhizophora apiculata* (Rambok et al., 2010). In Sundarban forests, the pH values were slightly alkaline ranging from 7.4 to 8.2 with various mangroves species (Das et al., 2012). For the salinity, there are a few species known as halophilic or salt tolerant (Nandy et al., 2007). Salinity may affect the structure of mangrove forests through the distribution of dominant species in Gazi Bay

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(Matthijs et al., 1999). Mangroves species that face tidal inundation daily or twice a month may tolerate extreme pH and high salinity (Nandy et al., 2007). But, Wakushima et al. (1994) stated clearly that the pH and salinity were highly affecting the zonal distribution of mangroves. *Kandelia candel* is the species that can grow well in low pH and salinity, while *Avicennia* sp., *Rhizophora* sp. and *Sonneratia* sp. can tolerate extreme pH and high salinity (Wakushima et al., 1994).

In term of soil chemical or nutrient properties, mangroves areas are also subjected to the changes of chemical concentrations, either in soil or water. Yet, the study about this matter is limited. Mangals are finely balanced, highly effective nutrient sinks with net imports of dissolved nitrogen, phosphorus and silicon. Nutrient fluxes in these environments are closely tied to particle size, pH and salinity, water input, plant assimilation and microbial mineralization (Alongi et al., 2013). Varying nutrient concentrations can also change competitive balances and affect species distributions (Chen and Twilley, 1999; Twilley and Chen, 1998). The carbon (C) in soil is determined by organic carbon and the production of C is depending majorly from litter such as leaf, propagule, seed, trunk and root (Alongi, 2014). The nitrogen (N) is highly present in soil through biological fixation, transformation and leaching (Nandy et al., 2007). Like C, N concentration in soil is also originated from the decomposition process of litter and the lack of this element in soil will cause soil infertility (Ashman and Puri, 2002). Phosphorus (P) element in soil is present as phosphate ion and it is required by the plant in large amount for growth (Greger, 2004). Potassium (K – known as Kalium) is another element that present in soil in form of cationic (Ashman and Puri, 2002). K is more abundant in silty or clayey soil compared to sand (Reef et al., 2010).

In mangrove forest, the nutrients are supplied by the litter fall from trees and suspended materials from surface runoff above ground, fauna activities and microorganisms' decomposition mechanism (Cannicci et al., 2008). The litter fall or surface materials were utilized by macro invertebrates such as *Scylla serrata* and that is broken down into smaller sizes. Then, the process was taken over by microorganisms until all the materials deposit into the soil permanently.

Soil physical and chemical properties of mangrove forest can indicate the current status and can determine the characteristics of tested soil (Furukawa and Wolanski, 1996; Havlin et al., 2014). Data obtained may represent the soil status of mangroves area thus to plan a proper action for enhancement of soil quality and governing ideal ecosystem. As medium of growth, soil should supply enough nutrients and have good characteristic to ensure better tree performance and establish greater forest ecosystem for wildlife conservation, economic value and most important to balancing environmental condition (Hopkins and Huner, 2004). According to Gruber and Galloway (2008), changes in land use patterns, coupled with climate issue and rising of global population have given a serious impact on nutrient release into the environment. Soil nutrient status has the most direct controls on the mangrove ecosystems (Alongi et al., 2003). Concentrations of nutrients and organic matter in mangrove soil can also be subjected to anthropogenic activities nearby such as sewage discharge either from and waste dumping (An et al., 2007).

As a result, nutrient availability could threaten ecological balance in mangrove ecosystems. Yet, the study about the effect of soil characteristics is still lacking in mangroves area. Many authors focused on the crop study as the intention to develop agricultural activities is the main concern for many countries (Ashman and Puri, 2002; Havlin et al., 2014). In this paper, we ask this question: to what extent the changes in

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land use pattern can alter the physical and chemical properties in mangrove soil? Hence, we attempt to examine seasonal and depth variation and variability of soil nutrient concentration in three different mangrove forests in Peninsular Malaysia, based on different anthropogenic activities.

Material and methods

Study site

In this study, three sites within Peninsular Malaysia were selected based on mangroves forest status and the activities at the surrounding area. The sites chosen are shown in *Figure 1*. The study was conducted within the Mangrove Forest Reserve (MFR) under the surveillance of Department of Forestry Peninsular Malaysia (DFPM). Generally, three criteria were selected; 1) mangroves at human settlement area (villages) at Kelantan (east coast), 2) mangroves at Ramsar's Site at Johor (southern-west coast) and 3) mangroves at agriculture area at Selangor (west coast).

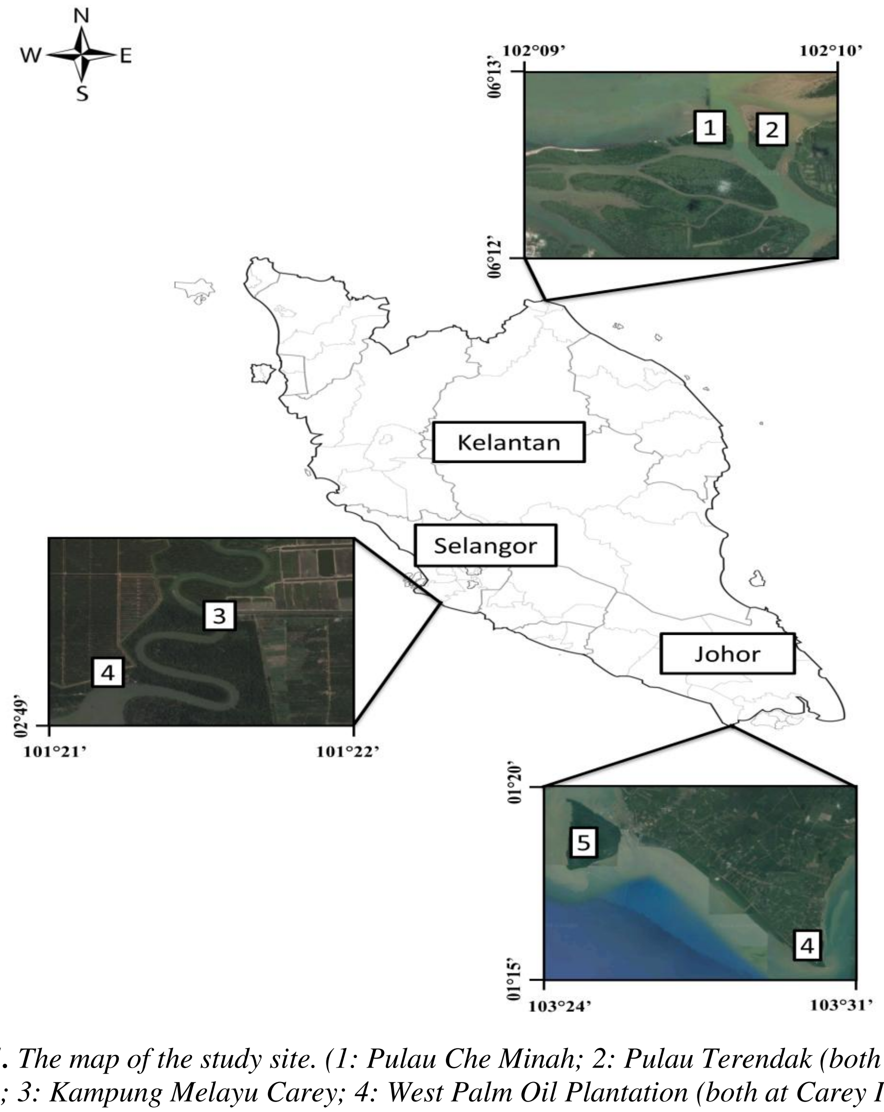

Figure 1. *The map of the study site. (1: Pulau Che Minah; 2: Pulau Terendak (both at Delta Kelantan); 3: Kampung Melayu Carey; 4: West Palm Oil Plantation (both at Carey Island); 5: Pulau Kukup; 6: Tanjung Piai (both at Ramsar's Site, Johor)*

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Delta Kelantan

The main river running through the delta is the Kelantan River, which meets the South China Sea at the east of the study area. A large delta has been developed by the river with sand spits and sand bars common at, and close to the outlets of larger river mouth. There are almost seventeen islands surrounding Delta Kelantan where area was covered with mangrove forests around 1200 ha. In this case study, Pulau Terendak (6º12'47.23" N 102º10'13.07" E) and Pulau Che Minah (6º12'44.38" N 102º09'53.84" E) were chosen and both locations were located in the western part of Kelantan Delta, Tumpat. Kelantan state’s climate regime is influenced by the northeast and southeast monsoons that blow from late November to March and from June to September respectively. The estimated total amount of rainfall was 2700 mm per annum with heavy rainfall during the Northeast monsoon (November to March). The temperature ranged from 22.7 °C to 32.9 °C with and estimated mean of 26.8 °C. The humidity of the region was estimated to be about 83.7 % (Malaysian Meteorology Department (MMD), 2010a; 2010b). There were few species can be found at Delta Kelantan. But the most common species were *Sonneratia caseolaris, Nypa fruticans, Avicennia alba, Rhizophora mucronata and Bruguiera gymnorrhiza* (Satyanarayana et al., 2010).

Ramsar sites, Johor

Johor is the southern states in peninsular Malaysia where also the home of three Ramsar Sites (wetland of Pulau Kukup, Tanjung Piai and Sungai Pulai Forest Reserves). Johor was estimated to hold 28.7 % (27,733 ha) of mangrove forests in Peninsular Malaysia (Wetlands International Malaysia, 2009). In Johor, Pulau Kukup (1°18'60.00" N 103°26'59.99" E), Tanjung Piai (01°16'1.177"N 103°30'37.766"E) and Sungai Pulai (1º23'39.01" N 103º32'33.3" E) besides being Ramsar Sites are also designated as State National Parks. Pulau Kukup, Tanjung Piai and Sungai Pulai covered about 10,299 ha of mangroves in Johor. Pulau Kukup is a mangrove island while Tanjung Piai and Sungai Pulai are coastal mangrove. In this study, only two from three Ramsar sites were chosen which are Tanjung Piai and Pulau Kukup. According to Tan et al. (2012), Pulau Kukup has the higher number of species with 11 species compared to Tanjung Piai with only 9 species. Pulau Kukup are typical examples of a *Rhizophora-Bruguiera* dominated coastal forest. It is dominated by *Rhizophora apiculata* as the most common species, followed by *Bruguiera cylindrica* and *Ceriops tagal*, while *Rhizophora mucronata* is found along channels. The total amount of rainfall in this area was about 2104.1 mm. The highest and the lowest rainfall were in July (302.9 mm) and April (84.4 mm), respectively. The air temperature was highly recorded in May with 29.5 °C and the lowest was in January 2011 of 26.0 °C (Nordatul Akmar and Wan Juliana, 2012).

Carey Island, Selangor

Carey Island, Selangor is located approximately 70 km south from Kuala Lumpur. Carey Island is one of the 8 islets within Klang Isles facing the Straits of Malacca and separated from mainland by Langat River. The total area of Carey Island is 16,187.45 ha comprising of 65 % of palm oil plantation while the rest are divided into forest reserves (1,876.85 ha) and settlements. The study was conducted at Kampung Melayu Carey (02°49'2.2376"N 101°21'16.236"E) and West Palm Oil Plantation (02°49'24.2904"N 101°21'34.65"E). The total annual rainfall is 2,100.5 mm with the

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maximum and minimum monthly rainfalls were 496.0 mm and 28.3 mm in December 2012 and March 2013, respectively. The tide at this area is irregular and semi-diurnal, with two high tides and low tides in one day. An extensive survey in 2008 revealed that there were 43 mangrove species including associate species fringing around Carey Island (Rozainah and Irfan, 2017). Furthermore, Saraswathy et al. (2009) recorded that *Avicennia alba, A. officinalis* and *Rhizophora mucronata* are the dominant species here. The zonation patterns of the mangrove trees are obviously dominated by *Avicennia* sp. and *Rhizophora* sp. majorly at 5 m to 20 m seaward zone.

Soil sampling

Soil samples at one-meter depth were taken during low tide using a soil sampler (Eijkelkamp multi-sampler) at each location with three replicates. Then, the soil was divided into five segments according to vertical depth (0-20, 20-40, 40-60, 60-80 and 80-100 cm). The soil samples were taken during three different seasons; wet season (October-December), dry season (June-September) and inter-monsoon period (March-May) from November 2012 until July 2014 (Malaysian Meteorology Department (MMD), 2010a, 2010b). The soil was kept in sealed plastic bag at 4 °C and brought back immediately to the laboratory for further analysis. In laboratory, the soil samples were air-dried in room temperature for five days. Then, the soils were sieved through 2-mm screen to remove visible roots, litters and large elements such as shell.

Soil physical (texture) and chemical analysis

The soil surface water was taken for *in-situ* salinity test using a refractometer (Master-S10M, Atago Co. Ltd., Tokyo, Japan) and the pH was tested using a pH meter (IQ 170, Spectrum Technologies Inc., San Diego, USA). A total of 270 soil samples were sieved into less than 2 mm and further tested using a Beckman Coulter (LS 13, 320) to determine the soil texture (silt, clay and sand). The results were then calculated by percentage while the soil triangle (USDA, 1951) was used to determine the soil type.

The soil chemical studies were conducted using four nutrient parameters. The organic carbon (C) content was analysed using the Walkley and Black analysis (De Vos et al., 2007) and the results were reported in percentage (%). The total nitrogen (N) analysis was done using Kjedahl method and results for the determination of total nitrogen by distillation process were reported in percentage (%) (Nelson and Sommers, 1980). Phosphorus (P) analysis was done by determining the available P in soil using the Deniege method and the results were reported in part per million (ppm) (Binkley and Fisher, 2012). The potassium (K) analysis was done using the determination of exchangeable K by distillation method and the results were reported in cmol/kg (Ross, 1995).

Statistical analysis

All the results from 2 sampling locations in each site were pooled together to represent only one site. Analysis of variance (ANOVA) was performed to compare differences in soil physical and chemical properties between study sites, seasons and vertical depth. Analyses were performed using SPSS (version 20.0), including the assumption tests. Means were compared using the Tukey honest significant difference (HSD) test to evaluate variations in physical and chemical properties with sites and seasons and with sites and depth.

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Results

Soil physical analysis

In terms of pH, Selangor and Kelantan showed that the surface water were slightly acidic in wet and intermediate season ranging from 6.4 to 6.9, while slightly basic in dry season with value >7. On the other hand, sites in Johor indicated that the water was in basic condition with value ranging from 8.3 to 8.7 across all seasons. The salinity results were difference for each site, where Selangor study site showed the highest value ranging from 20 ppm to 26 ppm, followed by Johor with value of 14 ppm to 16 ppm. The salinity at Kelantan was the lowest with the average of 11 ppm.

In this study, the soil textures were different for each location. The results showed significant difference amongst sites for all parameters. The composition of clay and silt were recorded high in Johor site while sand composition was highest at Kelantan. But in term of season, there were no significant difference for all locations. At Johor and Selangor study site, the soils were dominated by silty loam type. Whereas, in Kelantan, the soil type was sandy loam type (*Table 1*).

Table 1. *Soil texture at different seasons and locations (mean ± SD)*

Location	Season	Clay (%)	Silt (%)	Sand (%)	Soil type
Johor	Dry	9.24±0.65	75.34±3.46	15.40±3.69	Silt loam
	Wet	10.17±3.05	74.98±4.00	14.85±4.59	Silt loam
	Intermediate	9.12±0.30	71.84±2.95	18.80±3.22	Silt loam
	Average	**9.51±1.81^{x}**	**74.06±3.73^{x}**	**16.35±4.14^{z}**	Silt loam
Selangor	Dry	1.62±0.66	51.70±15.80	46.68±15.88	Silt loam
	Wet	2.19±0.63	52.27±14.53	45.55±14.83	Silt loam
	Intermediate	2.40±1.52	49.71±12.91	47.90±13.74	Sandy loam
	Average	**2.07±1.04^{z}**	**51.23±13.99^{y}**	**46.71±14.35^{y}**	Silt loam
Kelantan	Dry	4.93±1.78	39.74±15.26	56.15±15.55	Sandy loam
	Wet	3.30±2.11	24.69±16.80	72.02±18.88	Sandy loam
	Intermediate	3.85±1.85	28.12±15.95	68.01±17.82	Sandy loam
	Average	**4.02±1.98^{y}**	**30.85±16.78^{z}**	**65.39±18.20^{x}**	Sandy loam

^{x,y,z}Different letter denotes significant difference amongst locations

The detail results of the soil texture based on consecutive depth showed that different location had different trend. For example, in Selangor, the composition of clay increased going to the deeper segment. The result also showed that the clay in 60-80 cm significantly higher than 0-20 cm. In Johor, the results for silt and sand showed significantly difference between 0-20 cm and 60-80 cm. However, in Delta Kelantan, the soil texture showed no significant difference amongst depth (*Figure 2*). The soil types were identified at the end of the study. At Johor study site, the soil textures were silt loam for all depth. On the other hand, at Selangor study site, the soil types were different between 0-40 cm and 40-100 cm, sandy loam and silt loam, respectively. At Kelantan, the soil textures were identified as sandy loam except for 60-80 cm (*Table 2*).

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Soil chemical analysis

The results showed that there were significantly different in terms of carbon, nitrogen phosphorus and potassium for all locations. The results for carbon and nitrogen were following this order; Johor>Kelantan>Selangor. The result also showed that Selangor has the highest concentration of phosphorus, followed by Johor and Kelantan. For potassium, the result showed a different trend. The potassium concentration in Johor was significantly higher to the other locations. The following order is Johor>Selangor>Kelantan (*Table 3*). With exception for phosphorus in Johor study site (*Table 4*), all chemical properties according to consecutive depth were found to be not significant different.

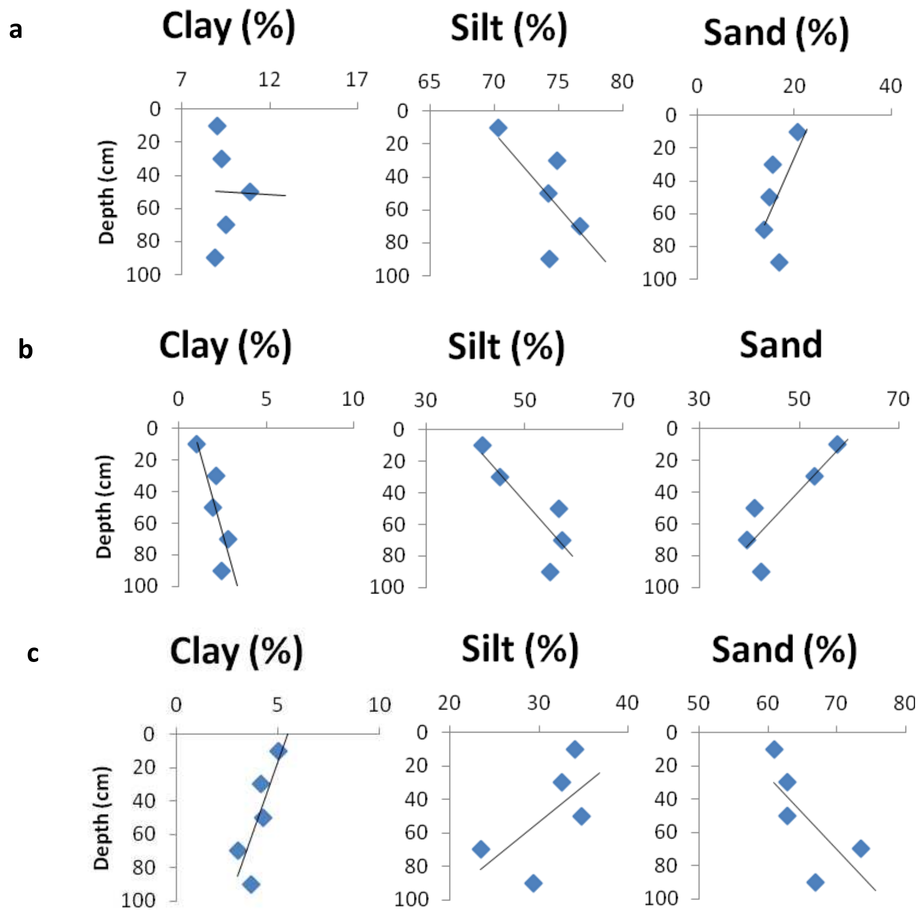

Figure 2. *Soil textures according to consecutive depth (a: Johor; b: Selangor; c: Kelantan)*

Table 2. *Soil texture based on consecutive depth (mean±SD)*

Location	Depth	Clay (%)	Silt (%)	Sand (%)	Soil type
Johor	0-20	8.99±0.37	70.33±3.62[b]	20.68±3.66[a]	Silt loam
	20-40	9.27±0.62	74.88±3.22[ab]	15.48±3.16[ab]	Silt loam
	40-60	10.87±3.84	74.15±2.66[ab]	14.92±3.97[ab]	Silt loam
	60-80	9.53±0.55	76.67±2.64[a]	13.80±2.60[b]	Silt loam
	80-100	8.90±0.61	74.24±4.20[ab]	16.87±4.41[ab]	Silt loam

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Selangor	0-20	1.02±0.36[b]	41.36±7.86	57.63±7.82	Sandy loam
	20-40	2.15±1.34[ab]	44.91±7.37	52.95±6.80	Sandy loam
	40-60	1.96±0.48[ab]	56.95±15.79	41.10±15.95	Silt loam
	60-80	2.79±1.07[a]	57.75±15.16	39.47±15.48	Silt loam
	80-100	2.43±0.94[ab]	55.18±16.36	42.39±16.76	Silt loam
Kelantan	0-20	5.01±1.93	34.11±11.80	60.88±13.71	Sandy loam
	20-40	4.17±2.21	32.52±19.60	62.85±21.93	Sandy loam
	40-60	4.25±1.83	34.79±19.76	62.75±18.95	Sandy loam
	60-80	3.01±1.96	23.46±17.35	73.53±19.43	Loamy sand
	80-100	3.69±2.05	29.36±17.78	66.95±19.81	Sandy loam

[a,b]Different letter denotes significant difference at different depth

***Table 3.** Soil chemical properties in different seasons and locations (mean±SD)*

Site	Soil depth (cm)	Soil nutrients			
		Carbon (%)	Nitrogen (%)	Phosphorus (ppm)	Potassium (cmol/kg)
Johor	Dry	5.18±3.62	0.27±0.34	25.74±6.05	2.83±0.72[a]
	Wet	6.91±3.58	0.20±0.23	25.24±6.07	2.01±1.12[b]
	Intermediate	6.59±1.82	0.13±0.04	25.28±5.09	2.50±1.80[ab]
	Average	**6.23±3.18[x]**	**0.20±0.24[x]**	**25.42±5.69[y]**	**2.45±1.32[x]**
Selangor	Dry	2.15±1.25[a]	0.05±0.01	38.55±16.70[b]	1.25±1.22
	Wet	1.45±0.63[b]	0.05±0.04	51.42±23.57[a]	1.22±0.30
	Intermediate	1.32±0.43[b]	0.05±0.02	31.53±9.29[b]	0.88±0.34
	Average	**1.64±0.91[z]**	**0.05±0.02[z]**	**40.50±19.20[x]**	**1.12±0.76[y]**
Kelantan	Dry	5.65±3.07[a]	0.11±0.047	23.79±12.90[a]	0.10±0.21
	Wet	1.62±0.69[b]	0.10±0.03	12.89±5.99[b]	0.27±0.53
	Intermediate	5.28±1.63[a]	0.11±0.03	18.76±8.27[a]	0.21±0.39
	Average	**4.18±2.73[y]**	**0.11±0.04[y]**	**18.48±10.40[z]**	**0.20±0.40[z]**

[a,b]Different letter denotes significant difference amongst seasons for each location
[x,y,z]Different letter denotes significant difference amongst locations

***Table 4.** Soil nutrients at different location according to consecutive depth (mean±SD)*

Site	Soil depth (cm)	Soil nutrients			
		Carbon (%)	**Nitrogen (%)**	**Phosphorus (ppm)**	**Potassium (cmol/kg)**
Johor	0-20	5.01±2.75	0.20±0.12	22.39±5.49[c]	2.34±1.05
	20-40	6.07±2.71	0.17±0.05	23.76±6.46[bc]	2.68±1.03
	40-60	6.97±4.10	0.29±0.42	24.70±4.05[abc]	2.24±1.04
	60-80	5.92±2.14	0.13±0.03	27.44±3.96[ab]	2.63±2.26
	80-100	7.16±3.64	0.20±0.30	28.79±6.02[a]	2.33±0.82
Selangor	0-20	1.49±0.34	0.05±0.02	40.04±14.97	1.12±0.61
	20-40	1.65±0.63	0.06±0.02	40.42±17.40	1.29±0.91
	40-60	1.79±1.07	0.05±0.02	38.39±23.12	1.11±0.64
	60-80	1.67±1.15	0.05±0.03	43.99±22.14	1.06±0.62
	80-100	1.61±1.14	0.04±0.03	39.63±18.96	1.00±1.00

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Kelantan	0-20	4.28±3.58	0.11±0.03	20.52±12.70	0.34±0.54
	20-40	4.07±2.73	0.11±0.03	21.04±9.47	0.21±0.50
	40-60	3.79±2.09	0.10±0.02	18.92±8.81	0.14±0.34
	60-80	4.16±2.26	0.10±0.20	15.16±10.21	0.01±0.11
	80-100	4.61±2.95	0.12±0.05	16.76±10.35	0.27±0.33

[a,b,c]Different letter denotes significant difference at different depth

Discussion

The mangrove soil or sediment condition and properties are very important in maintaining the integrity of mangrove ecosystem so that it can fully functioning while providing services to mankind and environment. A sound physical and chemical qualities would ensure that mangrove forest can thrive well despite of worldwide's physically challenging pressure on mangrove land. Malaysia is blessed with 60 % of forested mangroves which includes some areas that have been gazette as Permanent Reserve Forest (PRFs) and declared as Ramsar sites (Jusoff, 2013; Wong, 2004).

The pH value in Selangor and Kelantan was slightly acidic compared to Johor site. But those values which range from 6.5 to 9.0 are still within the suitable range for marine ecosystem (Robertson and Alongi, 1992). Similarly, the salinity values were within the range for mangrove to grow in healthy condition (Kathiresan and Bingham, 2001). Although the salinity level in Selangor was the highest compared to Kelantan and Johor site. According to Nandy et al. (2007), mangrove species that face tidal inundation daily or twice a month may tolerate extreme pH and high salinity. The site on Carey Island recorded high salinity 20-26 ppm. Carey Island experience daily tide range varies from 1.5 to 2.5 m and spring tides and neap tides occur twice a month. The site is exposed to high energy wind and currents, and shipping activities at the nearby Port Klang, whereby the current flow can go up to 70cm/s (Rizal et al., 2010; Sakmani et al., 2013). These winds generate waves with heights of 0.1 to 1.5m on the coasts of Carey Island with wave periods of 2~8 s (Muzathik et al., 2011). Kelantan showed very low salinity value, 11 ppm due to its deltaic settings. Johor site also recorded a low value with 14-16 ppm. Its location within a small and narrow Straits of Johor might impending a high salinity influence from the seawater.

Determination of the physical characteristics of the soil texture is important to further explain its ability to retain soil nutrients in the targeted area. Silt and clay particles are finer than sand and have a higher ability to trap nutrients (Ashman and Puri 2002; Kamaruzzaman et al., 2004; Nguyen et al., 2013). In our study, Johor study site recorded a finer soil particle compared to Kelantan and Selangor, with higher percentage of silt and clay compared and therefore, it is expected to retain more nutrients.

The sedimentation refers to the deposition of inorganic or organic matter onto the soil surface in two condition; allochthonous and autochthonous. Kristensen et al. (2008) explained allochthonous is the material derived from outside the mangrove area. For example, Sundarbans mangrove area received over billions of tonnes of sediment from Ganges-Brahmaputra River. Autochthonous refers to the material derived within the mangroves area such as leaf litter, dead trunks and branches, and roots (Cahoon et al., 2003; McKee, 2011). Delta Kelantan receive sediment from few large tributaries rivers that running down to the delta. Carey Island and Johor mainly receive sediment from within its mangrove forest.

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Furthermore, soil physical characteristics also influence primary soil nutrients such as carbon (C), nitrogen (N), phosphorus (P), and potassium (K) (Havlin et al., 2014). Many mangrove sediments have extremely low nutrient availability and high salinity, although they can vary greatly among and within mangrove forests (Reef et al., 2010). In contrast to most terrestrial soils, the flooded soils greatly restrain nitrification, the microbial formation of $NO3^-$, which would result in a low nutrient bioavailability in the intertidal sediments (Li et al., 2008). N deficiency (Feller et al., 2002, b; Reef et al., 2010) and salinity (Wakushima et al., 1994) have repeatedly been found to be important factors limiting mangrove productivity. Cheng et al. (2012) discovered that a moderate salinity could delay the entry of metals into the roots and thereby contributed to a higher metal tolerance.

The soil nutrients at Johor study site showed the highest concentration; i.e. CNK. But, for P, the value was lower than in Selangor. The soil results also showed a high concentration of nutrients in the bottom soil. This is followed what Havlin et al. (2014) discovered that more nutrients were concentrated in deep soil as a result of plants with extensive roots system, where the roots act as conduits for nutrients from the surface. Without any vegetation, nutrients were concentrated near the surface as a result of natural phenomena like weathering and atmospheric pressure (Jobbagy and Jackson 2001). A high concentration of nutrients, especially in the bottom soil, is very important so that they can be absorbed effectively by higher plants that have extensive root systems (Reef et al., 2010).

Mangroves also known as carbon-rich ecosystems that provide dumping pools for carbon from the atmosphere, allocthonous riverine, ocean elements and authochthonous organisms mechanism (Steven et al., 2004). C concentration was highest in the Johor and Kelantan sites, where those areas were not really affected by agriculture activities, unlike in Carey Island. According to Havlin et al. (2014), C is a fundamental element in mangroves as it promotes biological and physical health and protects from harmful elements. C concentration in the soil is largely determined by the volume of organic carbon as it is required in large amounts and can be obtained predominantly from the atmosphere. Soil organic carbon (SOC) is affected by both biotic and abiotic activities (Hopkins and Huner, 2004).

In this study, N also was highly concentrated at Johor and Kelantan. This is important because deficiency in N could result in soil infertility (Havlin et al., 2014). In soil, the ranges of N are about average of 0.05 % and the concentrations decrease with soil depth (Havlin et al., 2014). In our study, the N concentration at Selangor had the minimal effect to the mangroves growth. Some mangrove plants can accumulate the N through atmospheric nitrogen fixation or symbiotic bacteria present on or in the plants (Reef et al., 2010). The ammonium concentration is higher in muddy areas with high clay and silt composition compared to sandy soil (Alongi et al., 2013). Ashman and Puri (2002) described that the small particles such as silt have the stronger ability to retain N compared to sandy soil.

The P concentration was recorded high at Pulau Carey, Selangor. This is might be due to a small river running within Carey Island and receives sediments from the existing oil palm plantation activities as well as rainfall. The standard amount of P in soil must be within the range of 30-50 ppm (Havlin et al., 2014). For Johor and Kelantan, the values were below the minimum range. Potassium has also been used in fertilizer to increase soil fertility and acts as an indicator of healthy plants (Ashman and Puri, 2002; Havlin et al., 2014). The lack of K value in soil could lead to unhealthy plant growth. K is usually more abundant in silt or clay soil (Reef et al., 2010).

The current study found the amount of K was highly at Johor, followed by Selangor and Kelantan. At Kelantan study site, the value of K was within the range from very low to high concentration. The maximum values for K in soil should not less than 0.41 cmol/kg (Havlin et al., 2014).

Johor is expected to retain higher nutrient since this area was not really disturbed from anthropogenic activities, being gazetted under the Ramsar Site Treaty. This action is a very sound and exemplary of retaining mangrove forest so that little or none disturbance can be expected towards this site. And in turn, the mangroves forest at Tanjung Piai and Pulau Kukup were declared as Johor's State Park and proven that conservation status could well retain the quality and quantity of mangrove forest (Jusoff and Taha, 2008). Mangrove species differ in their growth-response to salinity. Our study sites recorded a reasonable value of salinity, as Patel et al. (2010) suggested that in extreme saline habitats N, P, K, Ca and Mg can be limiting factors for the growth of *A. marina.*

Conclusion

The soil in Johor and Selangor are dominated by silty loam while Kelantan soil is dominated by sandy loam. Nevertheless, the soil texture in these three study sites indicate stability in soil physical properties since there are no changes across seasons. In term of soil nutrient properties, the protected mangrove area in Johor has higher carbon, nitrogen and potassium compared to other sites. On the other hand, phosphorus concentration in Selangor soil is the highest which might be due to the run-off from oil palm agriculture site. Generally, there are no changes of nutrient concentrations across seasons except for carbon which is much higher in dry season in Selangor and Kelantan, while phosphorus level in Selangor is higher in wet season. Based on the depth, the nutrients are stable since there are no changes for all parameters except for phosphorus in Johor where it was found that the phosphorus was significantly higher in deeper soil.

The finding of this study indicates that the different mangrove habitats according to the land use types yielded different results in soil texture and nutrient composition. The protected mangrove habitat in Johor has siltier and clayey component which in turn can retain more nutrients for the plant growth. This finding proved that conservation and protection of mangrove habitats are needed to maintain the ecosystem services and functions for the well-being of mankind.

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ANALYSIS OF THE PROFITABILITY OF THE RESTITUTION OF FIRE-AFFECTED BEECH FORESTS IN SERBIA

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Abstract. The number of forest fires in Serbia caused by climate change has been continuously growing in recent decades, thus making the rehabilitation of the burned area increasingly important. In the period from 2003 to 2015, the total burned area (42.2%) was in beech forests. Cost-Benefit Analysis (the dynamic and the static approaches) was used as an approach to estimating the effects that restitution of beech forests can have on the environment, with the aim of finding the best solutions and making the best decisions about the desirability of the project. The dynamic approach (the analysis of tangible benefits and costs) led us to the conclusion that the investment in the restitution of beech forests destroyed by fires could be profitable only if it was based on wood production. Wood production may, depending on the site class, bear slightly higher interest rates (to better site classes) compared to previous estimates which ranged mainly around 3%. At lower discount rates (on poor quality sites), it takes more time to reach the break-even point, while the period of time needed to reach the break-even point shortens with higher discount rates. According to the static approach (the analysis of intangible benefits and costs) the benefits outweigh the costs, which makes the restitution of beech forests destroyed by fires acceptable.
Keywords: *forest fire, beech forests, restitution, financial analysis, economic analysis*

Introduction

Forests have always been considered as national treasures of a country. In addition to their ecological role in the preservation of important life cycles, they have other relevant economic and social (tourism, recreation, health) functions. Most of forest functions and their ecosystem services are not fully "captured" in comercial market (Costanza et al., 1997). This situation produce lack of valuation of ecoystem services what directy influence in sustainability of humans in the biosphere. There are many reasons for quoetionable in sustainability but one of most citet tiday can be found in processes of climate change. Forests are particularly vulnerable to climate change because they are not easily adapted to new environmental conditions.

The effects of modern civilization on climate change are becoming increasingly apparent. There has been an obvious trend of global temperature rise of 0.8°C since 1900 (Hansen et al., 2010). According to the European climate change scenario for 2100, the temperature is likely to rise by 2.8°C in Ireland and the UK, by 3.8°C in Central Europe and 4-5°C in Southern Europe (Christensen et al., 2007).

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The number of forest fires caused by climate change has been continuously growing in recent decades, thus making the rehabilitation of the burned area increasingly important. This number ranges between 50,000 and 70,000 fires a year in Europe. They affect 3,000 to 5,000 km^2 and cause millions of euro worth damage. Forest fires cause significant environmental, economic and social issues in many European countries, with possible long-term effects on the natural environment and the economy.

Climate change increases the risk of forest fire occurrence and spread (Allen et al., 2010; Ertuğrul and Varol, 2016). There are a large number of models and official reports that predict that the risk of forest fires will be increasing in the future (Flannigan et al., 2009; Aleksić et al., 2009; Sekulić et al., 2012). The climate, with the doubled amount of carbon dioxide in nature, will extend the fire season and increase the frequency and intensity of forest fires (Lindler et al., 2010). These predictions are so serious that they call for urgent changes in the forest fire management and the establishment of modern fire protection organization.

The total area of forests in Serbia amounts to 2,252,400 ha, or 29.1% of its territory (Banković et al., 2009). The state-owned forests account for 53.0% and privately-owned for 47.0% of the forested area. High intensity and frequency of fires may affect the sustainable management of forests to a certain extent. There is a general concern that the territory of Serbia has been affected by an increasing number of forest fires and this trend can cause substantial losses to forestry. Serbia has already recorded an increase in the frequency, intensity and duration of droughts. This trend will in the near future be particularly expressed in the southeast and east of Serbia.

The vulnerability of forest ecosystems depends on the ability of natural ecosystems to resist adverse influences (Schröter et al., 2005). Forest fires affect the forest ecosystem, and often seriously hinder its functioning over a longer period before it is restored to the state before the fire (González-Cabán, 2013). Proces of revitalization forest ecosistems is a matter of making the right choises, espoecialy from the aspect of income effect (Broberg, 2007).

Since revitalization of forest ecosystems requires significant financial funds, these sites are often left to natural regeneration. On the other hand, it is questionable whether the investment into the establishment of forest stands with the species that used to grow at these locations is reasonable at all.

In Serbia, in the period from 2003 to 2015, the total burned area (42.2%) was in beech forests. In this situation, the restitution of these forests after they have been damaged by forest fires is of huge economic importance. The process of forest restitution raises the issue of financing and assessing the profitability of the investment.

This problem can be viewed from many different aspects, but the main questions to be answered are:

- What will be produced and how much of it, i.e. which products will carry the greatest burden of repaying the financial liabilities?
- What discount rates should be applied in the assessment of profitability?

The main goal was to make an economic and financial analysis in order to examine the feasibility of the restitution of beech coppice forests, with the maximum considersation of ecological site characteristics.

It should be noted there has been no research related to the evaluation of the economic effeciency of investments in the artificial restitution of fire-damaged forest complexes (i.e. in the establishment of plantation). Previous analyses were mainly focused on the

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assessment of the cost-effectiveness of the establishment and exploitation of commercial plantations (artificially-established forest plantations) of certain genera of deciduous trees or on the evaluation of the investments in forest fire prevention.

For instance, Keča et al. (2009) applied some of the basic methods of dynamic assessment of the economic effeciency of investments (simple rates of yields, payback period, internal rate of return, net present value of the investment and cost-revenue ratio) in order to evaluate the investment in the establishment of an artificially-established plantation of one of the poplar clones in Serbia, with the subsequent commercialization of wood mass (tree cutting) 29 years after the establishment. They concluded that in the current financing conditions (at a fairly high cost of borrowing in the financial market) such an investment would not be commercially viable for any of the applied parameters. The same authors came to a similar conclusion in the case of the establishment of a commercial poplar plantation with a 25-year rotation period, with the recommendation that profitability would be achieved by lowering the capital costs to below 10%, i.e. by reducing the rotation period to 20 years (Keča et al., 2008). Similar results, for high interest rate conditions, were obtained in the economic assessment of the plantation establishment in Latvia (Greğe-Staltmane and Tuherm, 2010).

On the other hand, the evaluations of the economic effeciency of investments in forest fire prevention measures, regardless of the method used, showed a certain level of economic viability (Mokhtari et al., 2017). Moreover, recent years have seen the development of risk assessment models and economic assessment of the management of forest fire risk which are of particular interest in the regions that are highly endangered by forest fires (Rodriguez y Silva and Gonzalez Caba, 2010; Gould et al., 2013; Calkin et al., 2014).

Materials and Methods

Given that the investment goal should be the highest possible level of the obtained economic effects per unit of the invested financial funds, the level of the effects depends on the quantity and quality of both the expenditures and the revenues. The economic effeciency of the investment is calculated as the ratio of the obtained effects to the realized investments, or as a ratio of the realized investments to the obtained effects (Cicea et al., 2008), according to the equation (1):

$$e = \frac{E}{\varepsilon} \rightarrow maximum \text{ or } e' = \frac{\varepsilon}{E} \rightarrow minimum \quad \text{(Eq. 1)}$$

where e and e' represent economic effeciency; E is the obtained effect (the achieved result), while ε represents the realized investment (spent resources).

In the first case, the formula expresses the economic effect that is obtained per unit of the realized investment and it should have the maximum value. In the second case, the formula presents the investments realized per unit of the obtained economic effects and it should be minimum.

Cost-Benefit Analysis (with the Dynamic and the Static Approaches) was used to assess the economic effeciency of the realized investments. The value of production was calculated without taking into account exploitation costs because they would quite complicate the analysis (over longer periods of time they can significantly change with the changes in technology). Besides, there is a trend to separate forest exploitation from silviculture. The value of wood was calculated using the stumpage price list of the

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Public Enterprise “Srbijašume” (2012). Another reason for excluding the exploitation costs is the fact that the money invested in the exploitation cannot greatly change the amount and profitability of the invested funds because it remains tied up in the production for a relatively short time (Pudar, 1985).

The analysis included the following costs: coating stumps to prevent regrowth (purchase costs - 40,000 RSD per hectare) and cutting the shoots in the third year after the restitution had been conducted and the new stands established (22,400 RSD per hectare).

The subsidy granted by the state was treated as profit (for the organizations that perform the restitution). It amounted to 150,000 RSD per hectare and covered the costs of soil preparation, the value of seedlings, planting costs, the costs of ploughing and weeding with the aim of establishing high forests.

The cost of buying forest land was not taken into account since the restitution of beech forests was planned to be carried out on the areas with well-defined ownership status.

The profit was analyzed through the value of wood assortments at certain ages. The data on wood yield and the relevant prices of wood assortments were used to make tables showing the value of wood of the studied tree species growing on the sites of different quality classes. The obtained values were discounted at the discount rate ranging from 2 to 10%, which produced several different “current values”, depending on the applied discount rate. The value of wood production was calculated for different lengths of the production cycle (from 20 to 140 years). All the calculated values are expressed per unit area (1 ha).

The value of wood assortments that were taken into account when calculating the value of the wood production was determined using the stumpage prices of sawlogs class I (5,783 RSD) sawlogs class II (4,728 RSD) sawlogs class III (3,917 RSD) mine timber (4,852 RSD), cordwood (3,595 RSD), pulpwood (2,655 RSD) and forest residue (2,175 RSD)[1].

Apart from the tangible costs and benefits, the Cost-Benefit Analysis included a separate presentation of the intangible costs and benefits. Unlike the first approach (the dynamic approach), the second one (the static approach) was focused on the impact of beech forests on biodiversity (including species, ecosystem and genetic diversity), on environment, on the community and on the economy. For this reason, the dynamic method of assessing the profitability of the investments (net present value[2] and internal rate of return[3]) was supplemented with the static method of determining the intangible costs and benefits (i.e. the ordinal scale of Cost-Benefit Analysis and the quantitative data processing were used).

Results and Discussion

Tangible benefits and costs

We analyzed the value of wood production for the observed tree species on sites of different quality classes (I to V), with different lengths of the production cycle (20-140 years) and at discount rates of 2, 3, 4, 5, 6, 8 and 10%, since it was found that wood

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[1] 1 EURO = 124.00 RSD

[2] Net present value represents the difference between the cash flows expected to be generated from the investment and the cash outflows needed for its acquisition and use in the initial stage of using the investment (moment $n = 0$) (Subić, 2010). An investment is considered to be economically justified if its net present value is not negative (NPV> 0), *i.e.* if the ratio between the inflows and outflows discounted at the initial moment of its use (n = 0) is greater than 0.

[3] The internal rate of return can be defined as the interest rate, where the amount of monetary income from investments discounted at a given calculation moment is equal to the outflow needed for the purchase and use of the investment, discounted at the same calculation moment (Subić, 2010). The investment is economically viable when the internal rate of return is greater (or at least equal) than the assumed calculative interest rate (discount rate) (ISR ≥ i).

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production in forest cultures can be cost-effective only at discount rates lower than 10% (Brumelle et al., 1991).

In order to get a better insight into the flow of the current value of wood and the costs over time, we constructed graphs (*Figs. 1, 2, 3*) which show the curves of change in the current value of the wood of the given species at different site classes, depending on the length of the production cycle, for the six discount rates. The analysis of the data for all site classes shows that the investment in some site classes can be profitable even at slightly higher discount rates provided that the production cycle is short.

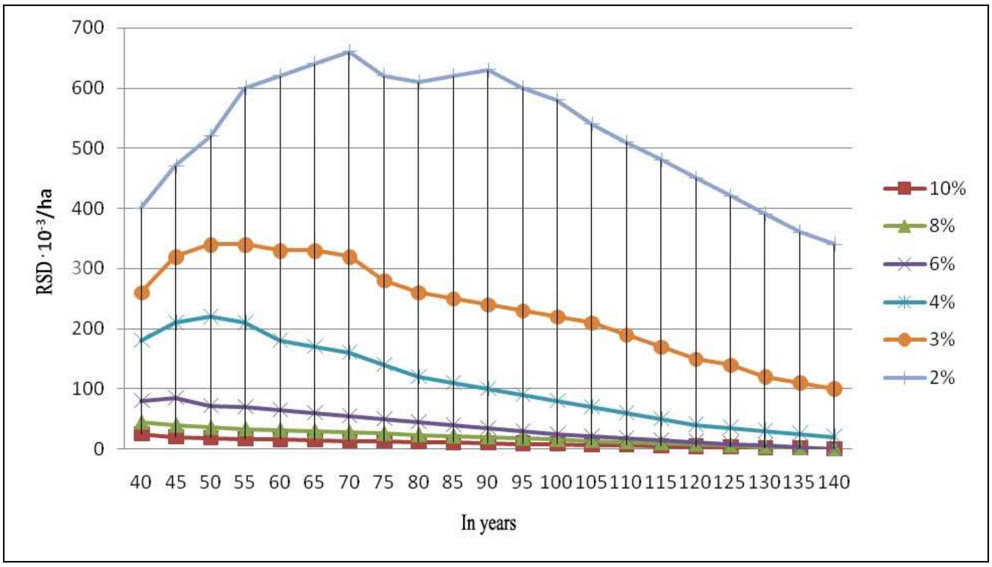

Figure 1. *Changes in the current value of wood with the age at different discount rates (Beech Site class I).*

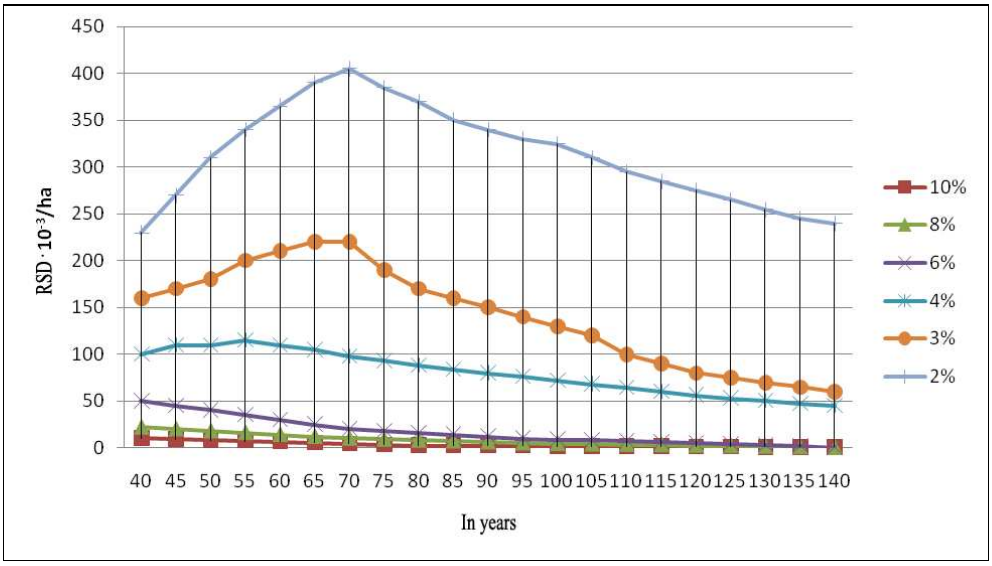

Figure 2. *Changes in the current value of wood with the age at different discount rates (Beech Site class III).*

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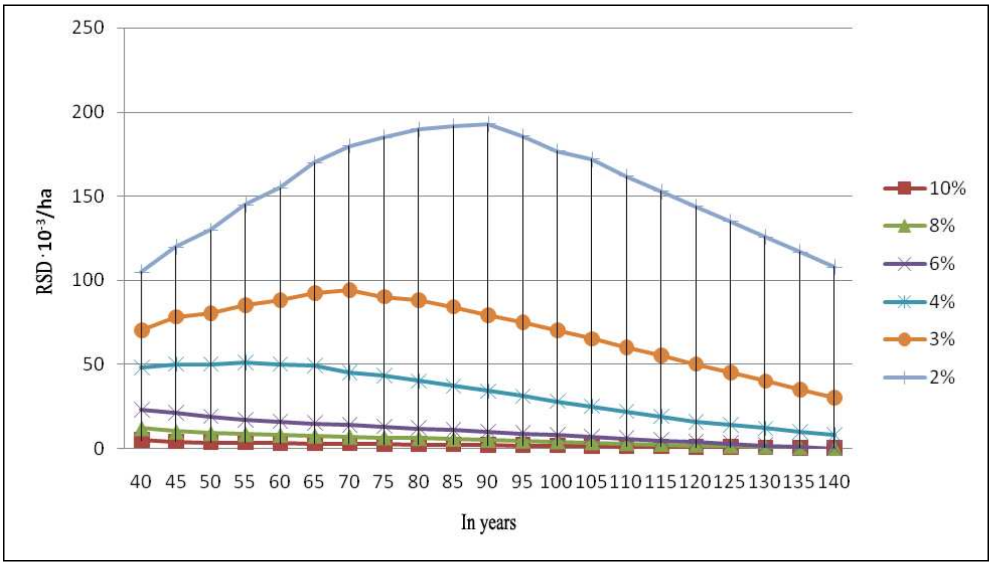

Figure 3. *Changes in the current value of wood with the age at different discount rates (Beech Site class V).*

Having in mind the equality of benefits and costs, we determined the highest discount rate and the maximum length of the production cycle at which the project could be expected to break even (*Table 1*). The table clearly shows that the discount rates at which the investment profitability is achieved are pretty lower than usual for similar projects (e.g. in agriculture). This indicates that wood production cannot bear regular interest rates. This fact should be taken into consideration when choosing the most appropriate interest rate.

Table 1. *The maximum discount rates and the break-even point by site classes.*

Site class	Discount rate (%)	Break-even point (in years)
I	5.66	45
II	5.19	50
III	4.28	55
IV	3.74	55
V	2.83	70

Source: Original

It can be concluded that if wood production is selected as the main production objective, the profitability of the investment can be achieved at different discount rates depending on the site class and the length of the production cycle (*Table 2*).

The obtained maximum discount rates are related to the length of rotation. In most cases, an increase in the discount rate shortens the length of rotation. This means that if long rotations must be selected from the aspect of the goal that is to be attained, the profitability can be achieved only at a discount (interest) rate below the maximum value.

This points to the complexity of the problem. There are no easy solutions and each particular case requires a thorough analysis to decide on the best option which would meet both financial and economic objectives.

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In some cases (site class and rotation), the profitability of investment can be achieved at higher discount rates (4%). This, to some extent, changes the current standing that investments in forestry can generally be profitable only at interest rates equal to or lower than 3%. Higher interest rates can mainly be applied only to better site classes (I and II), while the interest rate for poorer site classes (III-V) ranges around the values of 3% or even less than that (*Table 2*).

Table 2. *Break-even point of the production of wood in beech forests at different discount rates (per age).*

Discount rate (%)	Site class I	Site class II	Site class III	Site class IV	Site class V
2	Over 140	Over 140	Over 140	Over 140	140
3	135-140	120-125	110	95-100	-
4	85-95	75-80	70-75	-	-
6	-	-	-	-	-
8	-	-	-	-	-
10	-	-	-	-	-

Source: Original

It follows that the appropriate discount rate should be calculated for each particular case. The rate should correspond to the given tree species, site class and the length of the production cycle. The obtained value can be used to assess the profitability of the investments into the restitution of fire-affected beech forests.

”The duration of the production cycle” is in this case difficult to determine because the restitution of beech forests involves natural regeneration and production of wood and other forest benefits which can continue in perpetuity without any new investments. This is the fundamental difference between the forestry and standard industrial or agricultural projects, which in most cases have clearly defined duration, after which new investments are required.

Intagible benefits and costs

Apart from the dynamic method of determining the tangible benefits and costs, the static method of non-angible costs and benefits was also used. This method aims at determining the benefits that the restitution of beech forests brings to the wider community, but which cannot be expressed in monetary terms. To assess the intangible costs and benefits we used the ordinal scale of Cost-Benefit Analysis and the quantification (arithmetic operations) was done using the transformations that allow such procedures (Hastie et al., 2013). The results quantified on the basis of scoring the intensity of individual effects were used to perform the transformation (Ratknić and Braunović, 2013). The intensity of the effects and scoring are shown in *Table 3*.

Table 3. *Assessment of intangible effects on the environment.*

Effect	Intensity	Score
Positive or negative	Very weak	0-2
	Moderate	3-5
	Significant	6-8
	Very strong	9 – and over

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The process of restitution of beech forests is in the analysis divided into two periods: a period up to 20 years of age of new stands and a period between 21 and 120 years of age, when the rotation of beech high forests is completed. We selected 33 intangible effects (benefits and costs) that make relevant factors in the restitution of beech forests destroyed by forest fires (*Table 3*). The data are presented collectively by type of activity in *Table 4*.

Table 4. *Assessment of intangible benefits and costs of the planned project using the quantitative method – effects on biodiversity.*

1	2	3	4	5	6
Effects by type of activity	**Intangible benefits and costs (effects)**	**Significance and type of effect**		**Transformation**	
		Scoring of the effects in the period after the amelioration (in years)			
		up to 20	**21-120**	**up to 20**	**21-120**
Effects on biodiversity (including species, ecosystem and genetic diversity)	on macromycetes	- 4	+ 5	0.106	0.136
	on lichens	- 4	+ 5	0.106	0.136
	on moss	- 3	+ 3	0.076	0.076
	on the vascular flora	- 10	+ 10	0.288	0.288
	on Rotatoria	- 3	+ 3	0.076	0.076
	on the fauna of earthworms	- 4	+ 4	0.106	0.106
	on the diversity of snails	- 2	+ 2	0.045	0.045
	on the harvestman fauna	- 2	+ 2	0.045	0.045
	on the diversity of insects	- 8	+ 3	0.227	0.076
	on the diversity of amphibians and reptiles	- 9	+ 10	0.258	0.288
	on the diversity of birds	- 9	+ 10	0.258	0.288
	on mammals	- 6	+ 6	0.167	0.167
	on the ecosystem diversity	- 10	+ 8	0.288	0.227
	on the habitat fragmentation	- 9	+ 10	0.258	0.288
Effects on the environment	ensuring the functioning of the water regime (hydrological function)	0	+ 9		0.258
	protection of water against pollution	0	+ 8		0.227
	protection against harmful emissions	0	+ 7		0.197
	regulation of soil composition and fertility (and erosion control)	0	+ 6		0.167
	effect on the microclimate	0	+ 4		0.106
	production of oxygen and purification of the atmosphere	+ 1	+ 5	0.015	0.136
	carbon binding to wood volume and humic substances formed under forest	+ 1	+ 10	0.015	0.288
	effect on the physical appearance of the scenery during exploitation	- 7	+ 8	0.197	0.227
	rehabilitation of devastated areas	0	+ 7		0.197
	protection against noise	- 3	+ 4	0.076	0.106

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1	2	3	4	5	6
Effects on the community	provision of recreational opportunities	0	+ 6		0.167
	social benefits (job opportunities)	0	+ 4		0.106
	effect on human health	+ 1	+ 8	0.015	0.227
Effects on the economy	provision of raw materials for processing capacities	0	+ 10		0.288
	introduction of additional economic activities	+ 1	+ 4	0.015	0.106
	construction of commercial properties of permanent significance	- 4	+ 5	0.106	0.136
	effect on other economic activities (tourism, hunting, etc.)	0	+ 8		0.227
	use of other forest products (forest berries, medicinal herbs, and mushrooms)	+ 1	+ 8	0.015	0.227
	uncovered infrastructure costs	- 2	+ 2	0.045	0.045

Source: Original

The estimate of intangible benefits and costs is based on the assumption that they will be actually achieved (although it may not always be a realistic option). The analysis of intangible benefits and costs shows that in the first 20 years after the restitution has been performed, the costs are much higher than the benefits (*Table 5*). The reason is that the initial phase of the restitution is carried out on the sites that have been completely destroyed by forest fires and before the normal functioning of the new ecosystem is established, the costs are higher than the benefits.

In the period from 21 to 120 years of age, when the rotation of beech high forests ends, the benefits exceed the costs, which means that restitution is justified (*Table 5*). Of course, it should be noted that in each case (micro-location) an analysis should be conducted to determine whether intangible costs exceed benefits.

Table 5. *Quantification of the value of the estimate of intangible benefits and costs.*

Effects by type of activity	**Up to 20 years of age**		**From 21 to 120 years of age**	
	Cost estimate	**Benefit estimate**	**Cost estimate**	**Benefit estimate**
Effects on the biodiversity	2.303			2.242
Effects on the environment	0.273	0.030		1.909
Effects on the community		0.015		0.500
Effects on the economy	0.152	0.030		1.030
Total	2.728	0.075		5.682

Up to 20 years of age	Score (Benefit – Cost)<0	0.075 - 2.728 = -2.653
From 21 to 120 years of age	Score (Benefit – Cost)>0	5.682 = 5.682
Total	Score (Benefit – Cost)>0	(5.682+0.075)-2.728=3.704

Source: Original

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Conclusions

Forests and their resources belong to the resources of the future because they are renewable, and with the help of science and technology, they can replace some exhausted natural resources which are certain to disappear from the Earth. The establishment of new and the improvement of existing forests would enhance other forest functions (benefits to climate, conservation, erosion control, ambient aesthetics, tourism and recreation). It would further increase the yield of other forest resources - wild berries, mushrooms, medicinal and aromatic plants, or improve hunting opportunities. All in all, the cumulative effect on the level of society would be significant.

The presented results lead us to the following conclusions:

- The investment in the restitution of beech forests destroyed by forest fires can be profitable only if it is based on wood production and different discount rates can be applied depending on the site class.
- Wood production may, depending on the site class, bear slightly higher interest rates compared to previous estimates which ranged mainly around 3%. This primarily refers to better site classes, while on poor quality sites it may fall below the limit of 3%.
- The interest rate and the assessment of investment profitability are also closely related to the length of the production cycle because the break-even point varies with the discount rate. At lower discount rates, it takes more time to reach the break-even point, while the period of time needed to reach the break-even point shortens with higher discount rates. The length of production cycle plays an important role in determining the investment profitability, especially regarding the relation between the length of the production cycle and production targets (type and quality of wood assortments produced).
- Based on the analysis of intangible benefits and costs it can be concluded that the benefits outweigh the costs (generally speaking), which makes the restitution of beech forests destroyed by forest fires acceptable.

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THE EFFECT OF BACTERIAL APPLICATION ON THE PRODUCTIVITY OF FABA BEAN (*VICIA FABA* L.) AND ITS MIXTURE WITH SPRING WHEAT (*TRITICUM AESTIVUM* L.) UNDER TWO AGROCLIMATIC CONDITIONS IN LITHUANIA

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Abstract. Recently there has been a renewed interest in the optimization and development of legume cultivation technologies in many European Countries. By choosing the species which fit well the local soil and weather conditions might be an excellent solution to obtain a high fodder and grain yield. Field trials with faba bean (*Vicia faba* L.) and faba bean spring wheat (*Triticum aestivum* L.) mixture were conducted to evaluate the effect of seed inoculation with *Rhizobium* strain on the productivity of faba bean and its mixture with spring wheat. The study was conducted at two sites, Joniškėlis Experimental Station (site I) and Vėžaičiai Branch (site II) of the Lithuanian Research Centre for Agriculture and Forestry. The findings obtained in site I revealed that the dry matter (DM) yield of crops at the pod development stage averaged 7575 kg ha^{-1} and the grain yield averaged 6067 kg ha^{-1}; the corresponding values for site II were 5684 kg ha^{-1} and 4937 kg ha^{-1}, respectively. In most cases, the content of nutrients - crude proteins, crude fat, crude fibre, neutral detergent fibre, and crude ash in the biomass was higher in site I. The faba bean – spring wheat mixture produced a higher grain yield and a higher metabolizable energy content in grains compared with the faba bean grown as a sole crop; other measured parameters did not reveal the superiority of the mixture over the sole faba bean. *Rhizobium spp.* application had a positive impact by increasing the above-ground biomass DM yield and grain yield in a sole crop as well as in a mixture with wheat.
Keywords: *legume, mixture, dry matter production, seed quality, grain yield, Rhizobium spp.*

Introduction

Due to the increasing production area of cereals and oil crops in many European Countries, as well as the introduction of modern plant protection means, the area under legume crops and their role in crop rotation systems significantly decreased (Pascual and Perrings, 2007). According to the statistics, the area under legumes in the EU countries has declined from 5.8 million ha (in 1960) to 1.7 million ha (in 2013). Soybean (*Glycine max* (L.) Merr.) is a dominant legume crop, representing 50 % of the global legume crop area and 68% of global production (Herridge et al., 2008). Meanwhile in Europe, common pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.) are the predominant legumes. However, in order to diminish the dependence on import material (particularly soya bean meal) from America Continent and increase

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biodiversity according to the "greening" programme, some legume species have good prospects in the coming decades. Accordingly, the legume species have a huge potential worldwide (Jensen et al., 2015). Legume cultivation and economic profit depend on numerous factors, including economic, political, climatic, and social ones in each region and country (Pilorge and Muel, 2016). Grain-legumes have many functional and nutritional properties both as feed and food (Voisin et al., 2014; Magrini et al., 2016).

Recently in Lithuania, as well as in other countries of temperate climate, the area under faba bean has been steadily increasing (Siddique et al., 2012). Despite the fact that faba bean is more sensitive to biotic and abiotic factors than other legumes, it has a higher capacity for N fixation, higher photosynthetic activity, thus forming higher above-ground biomass (Jensen et al., 2010). In Lithuania, faba bean is an important protein source for both food and feed (Jensen et al., 2010). To improve feed quality, legumes are often grown in a mixture with barley, oats and other cereals (Strydhorst et al., 2008). Such mixtures could sustain more stable yields, improve nutrient cycling in the soil, perform better in terms of resource use efficiency, provide better protection against weeds, diseases and pests, and increase biodiversity (McKenzie, 1999; Strydhorst et al., 2008; Šarūnaitė et al., 2013).

Legumes are not only a source of protein, they also play a significant role in the nitrogen cycle in the biosphere due to their most important ecological process - atmospheric nitrogen fixation (Bloemberg and Lugtenberg, 2001). The cultivation of faba bean and other legumes has an important economic benefit in sustainable cropping systems, which allows reduction of of mineral N fertilizer use, whose manufacturing requires fossil materials (Nemecek et al., 2008; Bedoussac et al., 2015). Each year, in agricultural systems, annual crops capture up to 50-70 Tg of atmospheric nitrogen (Herridge et al., 2008). The inoculation of faba bean seeds with *Rhizobium* strains has a positive effect on the symbiotic N fixation rate, above-ground biomass of crops as well as seed productivity (Lapinskas, 2006; Elkoca et al., 2007). *Rhizobium* bacterial activity is the most efficient in the soils that had been intensively managed, including heavy input of chemicals, for many years and no bacterial agents had been used for more than 10 years.

Inoculation of faba bean seeds is an efficient tool to increase crops productivity in a crop rotation and enhance economic profitability. Although the positive role of faba bean has been known for a long time, the growing technology needs to be refined and optimized. It has been well documented that inoculation of legume grains by bacterial strains is an efficient way of introducing effective *Rhizobia* to the soil and the rhizosphere of legumes. Threrefore, much work has still to be done in order to realize the full productivity potential of faba bean (Deaker et al., 2004).

The aim of the current study work was to get a better understanding and evaluate the efficiency of *Rhizobium* application on faba bean and faba bean – wheat mixture and their productivity in the two locations, differing in precipitation regime and soil type.

Materials and methods

Field experiments were carried out in two locations differing in the agroclimatic contitions: Joniškėlis Experimental Station (site I) and Vėžaičiai Branch (site II) of the Lithuanian Research Centre for Agriculture and Forestry. Joniškėlis Experimental Station is situated in the northern part of Central Lithuania's lowland (56°12′ N, 24° 20′ E). The soil of this experimental site is *Endocalcaric Endogleyic Cambisol (Siltic, Drainic)* (clay (<0.002 mm) – 27.0 %) with pH_{KCl} 6.5-7.0, P_2O_5 and K_2O - 198 mg kg^{-1}

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and 273 mg kg^{-1}, respectively, humus - 2.8-3.0 %. The annual amount of precipitation is 500-600 mm, and most precipitation falls in the spring – summer period. Vėžaičiai branch is located in the seaside lowland eastern edge (Western Lithuania, 55°43′ N, 21° 27′ E). The soil of this experimental site is naturally acid, moraine loamy *Bathygleyic Dystric Glossic Retisol* (WRB, 2014), (clay (<0.002 mm) – 15.0 %), pH_{KCl} 5.2-5.5, P_2O_5 and K_2O - 200 mg kg^{-1} and humus 2.2-2.3 %. The annual amount of precipitation is 700-900 mm and most of it occurs in the autumn and early winter period.

Experimental designs and details

The experimental site was divided into two strips (factor – crops) – faba bean as sole crop and faba bean – spring wheat mixture. Further, each strip was randomly split into two smaller strips (factor – *Rhizobium* application) - without *Rhizobium* application and with *Rhizobium* application. The plots were sowin at the rates of 0.65 million ha^{-1} (65 faba bean plants m^{-2}) of viable faba bean seeds (in sole crop) and 0.40 million ha^{-1} of viable faba bean seeds + 2.5 mlillion ha^{-1} viable spring wheat seeds (40 faba bean plants m^{-2} and 250 wheat plants m^{-2}). Faba bean seeds (cv. Bioro) were inoculated with microbial fertilizer *Rhizobium leguminosarum bv. fabae* just before the sowing. The rate of phosphorus (single superhosphate) was equal for all treatment – 60 kg ha^{-1} P_2O_5. other fertilizers have not been used. Fertilizers were incorporated into the soil with a combined seed drill together with seeds.

Plant analyses

The dry matter yield was assessed at a pod development stage on July 18 and July 16 in site I and site II, respectively and at a full maturity stage (grain yield adjusted to 14% moisture) on August 20 and August 11 in site I and site II respectively.

The following chemical parameters were analysed: crude proteins (CP) (after Kjeldahl), crude fat (CF) (Soxhlet extraction method), water-soluble carbohydrates (WSC), crude fibre (Cfib) (by Henneberg and Stohman method), neutral detergent fibre (NDF) (by Van Soest method) and crude ash (CA) (gravimetrically). All analyses were done at the Chemical Laboratory of the Lithuanian Research Centre for Agriculture and Forestry.

The data of all studied parameters and their interactions were statistically processed using analysis of variance (*ANOVA*) as a three factorial randomized block variant to determine significant differences between means (*P< 0.05 and **P< 0.01), LSD_{05} and LSD_{01} (at 95 % and 99 % probability levels) (Tarakanovas and Raudonius, 2003).

Results and discussion

According to the data of Fisher's criterion, at pod development stage, location/site (L) had an essential effect on all studied parameters: dry matter yield (DM kg ha^{-1}), crude proteins (CP), crude fat (CF), crude fibre (CFib), crude ash (CA), water soluble carbohydrates (WSC), neutral detergent fibre (NDF) contents (mg kg^{-1}) and energy value (MJ kg^{-1}) (EV) (at p< 0.01) (*Table 1*). There were no significant differences in DM yield between the both crops (Cr) (growing faba bean as a sole crop and in mixture with spring wheat). However, there were significant differences (at p< 0.01) in all the studied chemical parameters as well as energy value beteween the both crops. Bacterial application (Rh) caused the differences between crops' DM yields (at p< 0.05), CF, CFib, NDF contents as well as ME (p< 0.01). There were significant differences

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between location and crop (LxCr) interaction in the following parameters: CF, CFib, CA and ME (at p< 0.01). The effect of location (L) and bacterial (Rh) interactions on the studied parameters (except insignificant effect on CA content) was similar. CrxRh interaction caused the differences in CF, Cfib (at p< 0.01) and CA (at p< 0.05) contents. With regard to the three factors' interaction between LxCrxRh, it gave a significant impact on CP, CF and CA contents in the above-ground biomass (at p< 0.01).

Table 1. *F statistics values for the studied parameters at pod development stage*

Source of variation	DM	CP	CF	WSC	CFib	NDF	CA	ME
Loc. (L)	46.94**	1024.88**	329.87**	148.94**	79.93**	140.88**	94.19**	225.47**
Crops (Cr)	1.61	46.81**	101.51**	33.78**	31.84**	83.6**	15.12**	91.76**
Bact. (Rh)	6.15*	0.64	324.91**	0.04	13.66**	7.80**	0.53	21.33**
LxCr	0.48	1.84	213.11**	1.58	7.09*	0.93	7.90**	40.78**
LxRh	2.08	0.96	221.19**	0.07	23.50**	0.44	0.12	24.35**
CrxRh	0.34	0.10	339.91**	0.49	8.41**	1.28	5.75*	31.48**
LxCrxRh	0	1024.88**	514.65**	0.69	0.23	3.22	9.51**	34.71**

Note: DM - dry mater, CP - crude proteins, CF - crude fat, WSC - water soluble carbohydrates, CFib - crude fibre, NDF - neutral detergent fibre, CA - crude ash.

At a full maturity stage, the location/site (L) as a factor had an essential effect on the grain yield (GY) and grain chemical parameters: CP, CF, CFib, CA, WSC and ME (at p< 0.01) (*Table 2*). There was no difference between crops (Cr) SY; however, grain quality parameters as well as EV differed significantly (at p< 0.01). Bacterial application (Rh) had an impact on SY (p< 0.05) and the following parameters: CF, CFib, WSC, NDF and ME (at p< 0.01).

Table 2. *F statistics values for the studied parameters in grain yield and seed quality parameters*

Source of variation	GY	CP	CF	WSC	CFib	NDF	CA	ME
Loc. (L)	29.12**	46.29**	6.12*	33.33**	109.19**	2.75	104.4**	0.89
Crops (Cr)	1.06	348.69**	26.53**	310.64**	63.70**	99.98**	86.20**	606.68**
Bact. (Rh)	7.39*	0.63	110.34**	40.44**	14.96**	4.71*	2.72	61.08**
LxCr	8.72**	244.4**	4.62*	4.32*	0.31	20.73**	6.85*	0.27
LxRh	0.11	0.13	2.50	44.08**	21.83**	1.52	5.52*	1.17
CrxRh	0.88	0.42	0	3.80	5.34*	5.48*	0.34	246.93**
LxCrxRh	0.11	2.31	0.59	1.86	12.07**	0.80	29.35**	1.64

Note: DM - dry mater, CP - crude proteins, CF - crude fat, WSC - water soluble carbohydrates, CFib - crude fibre, NDF - neutral detergent fibre, CA - crude ash.

Concerning the interactions among the different factors, LxCr was the most distinctive and strongest for the studied parameters: GY, CP, NDF and ME (at p<0.01) as well as for CF, CA and ME (at p< 0.05). The effect of the other two pairs of interaction was less noticeable. In this respect, LxRh interaction had a significant variation for CFib, WSC (at p< 0.01) and CA, ME (at p< 0.05); meanwhile CrxRh

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interaction significantly influenced CFib and NDF (at $p< 0.05$). The three-fold factorial interaction (LxCrxRh) had a significant impact for CFib and CA (at $p< 0.01$) only.

The data of the above-ground biomass dry matter (DM) yield at pod development stage are presented in *Figures 1* and *2*.

The results evidently showed that the location/site (growing under different soil and temperature regime) had an essential effect on DM yield. The average DM yield at site I (Joniškėlis) surpassed DM yield at site II (Vėžaičiai) by 33.27 % (at 99 % probability level). In both locations, when growing faba bean in mixture with spring wheat, DM yield was higher than that in sole crop; however, the increase of biomass was not statistically significant (at 95 % level).

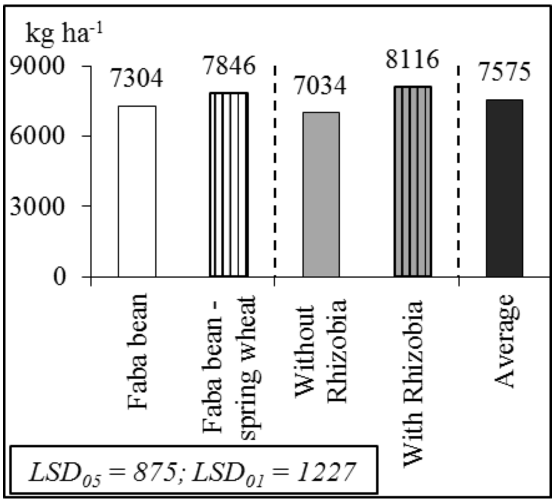

Figure 1. Faba bean and faba bean - spring wheat mixture (kg ha^{-1}) dry matter (DM) at pod development stage as affected by crop and *Rhizobium* application at site I

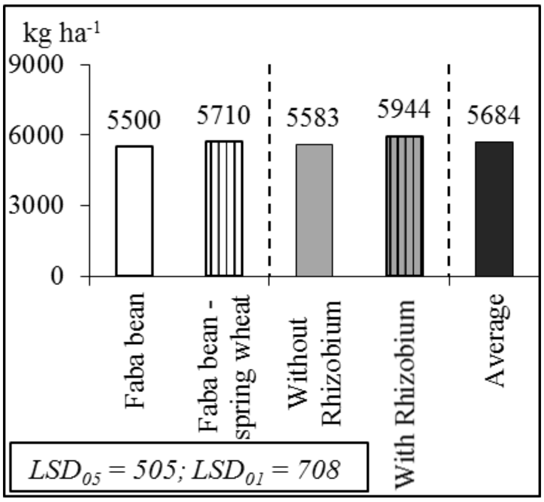

Figure 2. Faba bean and faba bean - spring wheat mixture (kg ha^{-1}) dry matter (DM) at pod development stage as affected by crop and *Rhizobium* application at site II

The inoculation of seeds with of *Rhizobium* had a significant and positive effect on biomass productivity. DM yield increased from 7034 kg ha^{-1} to 8116 kg ha^{-1} (by 15.38 %) (in site I) and from 5583 kg ha^{-1} to 5944 kg ha^{-1} (in site II) (both at 95 % probability level).

The highest grain productivity was obtained at site I where the yield averaged 6067 kg ha^{-1} and was by 22.89 % higher than in site II) (*Figs. 3* and *4*).

The grain yield in the faba bean – spring wheat mixture amounted to 6268 kg ha^{-1}, which was by 6.87 % higher than of sole bean crop (site I) (at 99 % probability level). However, the opposite situation was observed at site II, where sole crop produced higher yield averaging 5353 kg ha^{-1}. As mentioned before, the sowing rate of faba bean to be grown in a mixture with cereals was lower compared to that of a sole crop. Further, the spring wheat plants were shorter than faba beans ("Bioro") (biometrical data are not presented), therefore beans shaded them, which resulted in a relatively smaller share of wheat plants in the mixture.

The application of *Rhizobium* resulted in a grain yield increase from 5817 kg ha^{-1} (without inoculation) to 6316 kg ha^{-1} (by 8.58 %) at site I and from 4617 kg ha^{-1} to 5257 kg ha^{-1} (by 13.86 %) at site II (both at 99 % probability level).

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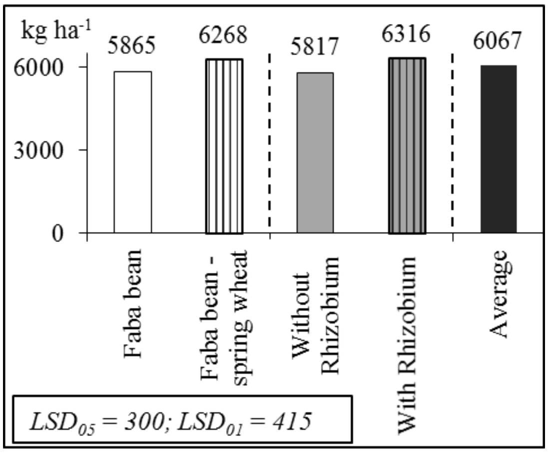

Figure 3. Faba bean and faba bean - spring wheat mixture (kg ha^{-1}) grain yield (14% moisture) at full maturity stage as affected by crop and Rhizobia application at site I

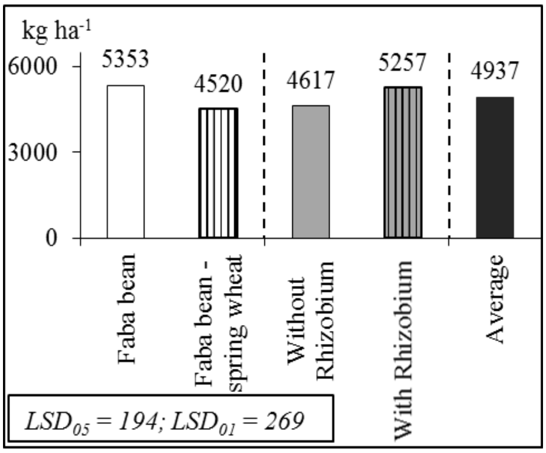

Figure 4. Faba bean and faba bean - spring wheat mixture (kg ha^{-1}) grain yield (14% moisture) at full maturity stage as affected by crop and Rhizobia application at site II

The data of chemical parameters of faba bean and faba bean – spring wheat biomass at faba bean pod developing stage are presented in *Table 3*.

Table 3. The contents of nutritional parameters in the above-ground biomass (CP, CF, WSC, CFib, NDF, CA (g kg^{-1}) and metabolizable energy (MJ kg^{-1}) content at pod development stage

Variables	**CP**	**CF**	**WSC**	**CFib**	**NDF**	**CA**	**ME**
Site I							
Faba bean	184	24.25	101.2	426	483	82.67	19.58
Faba bean + spring wheat	172	28.75	81.17	414	524	80.83	19.51
Without *Rhizobium*	180	29.50	91.83	438	509	80.83	19.59
With *Rhizobium*	176	23.50	90.50	402	499	82.67	19.50
Average	178	26.50	91.17	420	504	81.75	12.71
$LSD_{05/01}$	*3.43/ 4.81*	*0.22/ 0.31*	*6.47/ 9.07*	*8.26/ 11.58*	*10.08/ 14.13*	*2.16/ 3.03*	*0.020/ 0.028*
Site II							
Faba bean	113	23.60	172	400	419	70.95	19.01
Faba bean + spring wheat	93.9	22.76	170	365	470	59.57	18.94
Without *Rhizobium*	103	23.48	169	380	453	64.93	19.01
With *Rhizobium*	104	22.90	174	385	436	65.58	19.01
Average	104	23.19	171	383	444	65.26	12.68
$LSD_{05/01}$	*3.39/ 4.75*	*0.24/ 0.34*	*1.24/ 1.74*	*5.19/ 7.28*	*4.87/ 6.82*	*1.85/ 2.59*	*0.017/ 0.024*

Note: CP - crude proteins, CF - crude fat, WSC - water soluble carbohydrates, CFib - crude fibre, NDF - neutral detergent fibre, CA - crude ash, ME - metabolizable energy.

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In most cases, (except for WSC), the average values of quality parameters,(CP, CF, CFib, NDF and CA) were higher at site I (Joniškėlis).

At site I, the contents of CP, WSC, CFib and CA in sole faba bean biomass were significantly higher than those in faba bean – spring wheat mixture by 6.98 %, 24.68 %, 4.68 %, 2.90 % and 2.28 %, respectively; altogether, CF content in the biomass mixture was higher by 8.49 %. At site II, the higher contents of nutrient parameters were in sole faba bean biomass as well, where CP content was by 20.34 %, CF by 3.69 %, WSC by 1.18 %, CFib by 9.59 %, CA by 19.10 % higher than in the faba bean – wheat mixture. Only the content of NDF was higher in the mixture by 10.85 %.

In many cases, the role of *Rhizobium* on the studied parameters was unambiguous; however, the application of bacterial application had no positive effect on the measured quality parameters.

As regards the amount of metabolizable energy, there were no significant differences between either the two sites or *Rhizobia* application. However, the share of the energy was higher in the faba bean sole crop than in the faba bean – wheat mixture.

Faba bean and faba bean – spring wheat chemical parameters in grains at full maturity stage was somewhat different from that at pod development stage (*Table 4*).

Table 4. The contents of nutritional parameters in faba bean – spring wheat grains (CP, CF, WSC, CFib, NDF, CA (g kg^{-1}) and metabolizable energy (MJ kg^{-1}) content at full maturity stage

Variables	CP	CF	WSC	CFib	NDF	CA	ME
Site I							
Faba bean	271	15.75	65.50	102.33	150	38.17	13.21
Faba bean + spring wheat	264	13.50	55.33	88.50	140	34.83	12.21
Without *Rhizobia*	269	13.25	64.67	94.67	148	37.50	12.89
With *Rhizobia*	266	16.00	56.17	96.17	142	35.50	12.54
I location	267	14.62	60.42	95.42	145	36.50	11.79
$LSD_{05/01}$	*2.16/ 3.02*	*0.355/ 0.498*	*0.74/ 1.03*	*2.54/ 3.57*	*3.64/ 5.10*	*0.90/ 1.26*	*0.067/ 0.095*
Site II							
Faba bean	291	14.32	63.08	83.90	162	34.37	13.16
Faba bean + spring wheat	212	13.40	50.20	67.98	134	28.42	12.19
Without *Rhizobia*	252	12.00	56.55	83.90	149	31.22	12.81
With *Rhizobia*	251	15.72	56.73	67.98	148	31.57	12.54
Average	252	13.86	56.64	75.94	148	31.39	12.14
$LSD_{05/01}$	*4.87/ 6.83*	*0.590/ 0.830*	*1.14/ 1.60*	*3.21/ 4.50*	*4.87/ 6.82*	*0.729/ 1.02*	*0.044/ 0.062*

Note: CP - crude proteins, CF - crude fat, WSC - water soluble carbohydrates, CFib - crude fibre, NDF - neutral detergent fibre, CA - crude ash, ME - metabolizable energy.

In general, the values of different grain nutrient parameters were higher at site I. However, these differences were less apparent than those in biomass at pod development stage. Overall, the content of nutritional elements in sole faba bean grain

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was by 2.65 – 18.38 % higher compared with faba bean – wheat mixture grains in site I and by 20.94 – 37.26 % higher in site II. Except for CF, *Rhizobium* application did not increase the content of the studied parameters. It is known that a clear inverse relationship exists between protein and fat content in grains of many crops; that is with decreasing CP content, the CF content increases.

Despite the lower nutritional elements content, the higher amount of metabolizable energy was estimated in the grain from site II. As well, higher amount of the energy, averaging 13.21 MJ kg^{-1} was estimated in sole faba bean grains in site I and 13.16 in site II, There is an inverse relationship between CFib and metabolizable energy. *Rhizobia* application tended to decrease metabolizable energy amount in grains.

Discussion

Up to now, the development of legume growing technologies has been lagging behind that of cereal crops (Siddique, 2012). Consequently, an increase in the productivity of legumes has also been falling behind so far. Since legume – cereal mixtures are important components in animal husbandry, we intended to compare a sole faba bean sward with a faba bean – spring wheat mixture. At pod development stage, the DM yield of the biomass of mixture tended to be higher than that of faba bean sole crop. However, the grain yield results are unambiguous. Contrary to our expectations, grain yield of the faba bean – wheat mixture was lower than that of sole faba bean (site II, Vėžaičiai). Yet, some authors suggest that depending on the crop mixture, competition for water and nutrients, the yields in some cases might decrease (Yadav and Yadav, 2001; Olowe and Adeyemo, 2009). Our research findings do not always agree with those reported by other authors. Some research evidence suggests that cultivation of two or more crops in a mixture has the potential to achieve higher grain yields than do sole crop systems by improving efficient use of water, light, and nutrients (Zhang et al., 2010; Arlauskienė et al., 2011). However, other authors argue that faba bean - cereal mixtures do not necessarily increase faba bean sward grain yield or biomass productivity (Scalise et al., 2015).

One of the most important factors, which limit crop productivity, is the N fixation efficiency. It has been documented that inoculated plants activate nitrogen fixation, increase the rate of photosynthesis and respiratory enzymes; therefore, plants produce higher yields (Lapinskas, 2008). Legume bacteria sensitively react to a wide range of environmental conditions, including moisture, aeration, temperature and lighting variations (Köpke and Nemecek, 2010). Among other legumes, faba bean yield highly depends on N fixation efficiency (Jensen et al., 2010). N fixation efficiency as well as faba bean yields highly vary (Jensen et al., 2010; Foyer et al., 2016). In dry vegetation periods, the limiting biotic factor in the upper layer of *Cambisols* is high amount of clay particles, which leads to low aeration and crust formation. Still, clay soils are characterized by a weaker microbial activity and less favourable conditions of soil fauna (Janušauskaitė et al., 2008). In turn, *Albeluvisols* are traditionally acid soils, in which mobile aluminium and its toxic effect on plant roots are important limiting factors for many traditional crops. In both cases, taking into account these yield limiting factors, faba bean productivity might be increased by the application of the following practices: seed inoculation with bacterial products, optimization of the basic soil tillage, crop rotation diversification of basic and catch crops (Verhulst et al., 2010). These

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experiments confirmed the positive effect of *Rhizobia* bacteria on DM increment as well as grain yield at both experimental sites.

Another issue we addressed in our research was chemical parameters of the biomass. In particular, faba bean plants are valued as protein feed (Voisin et al., 2014), which is required for weight gain of ruminants, milk production, maintenance of vital and reproductive functions (Butkutė, 2011) and are also suitable for monogastric animals (Jezierny et al., 2010) and laying hens (Koivunen et al., 2014). We found that there are few published data on how well the bacterial fertilizer affects different nutritional parameters of legumes. We expected to emphasize the role of *Rhizobium* to nutrient composition. Although there were significant differences in the parameters between the both study sites, the direct effect of *Rhizobia* application on nutrient parameters in the biomass as well as in grains was less explicit. Ascertainment of the role of bacterial agents for chemical parameters of the biomass warrants more precise experiments. Moreover, further studies on the current topic are required to validate the positive role of legumes, particularly faba bean in agricultural systems and to more comprehensively elucidate the effects of *Rhizobium* (or other bacterial strains) on plants and their nutritional quality.

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SLIGHT SOIL DEACIDIFICATION COMPROMISE THE GROWTH AND THE ALUMINUM ACCUMULATION IN *Qualea cordata* PLANTS

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Abstract. Soil acidity and aluminum presence may reduce plant growth worldwide. In several cases, these two parameters, either alone or associated, can lead to plant constraints, which is barely known in natural ecosystems. In order to address these constraints, current model of study is *Qualea cordata*, an endemic species of Brazilian impoverished soil of rupestrian fields. Plants were grown, both in very acidic soil and in little deacidificated soil. After 6 months of growth in two acidic soil conditions and aluminum presence, the accumulation and dry biomass partitioning and content of nitrogen, calcium, magnesium, phosphorus, aluminum and chloroplastic pigments were evaluated and also chlorophyll fluorescence was obtained in intact plants. The soil deacidification induced an increase of mineral element contents in the leaves, however, lower total plant biomass and aluminum accumulation, also induced differential biomass partitioning and greater carotenoid levels. Regardless of the soil acidity, the levels of chlorophyll were not altered even in photosynthetic parameters. The results highlighted the resilience of this species to natural and very acidic soil conditions, indicating the need and the beneficial effect of aluminum accumulation in this specie.
Keywords: *aluminum resilience, metal accumulator, soil pH, Vochysiaceae*

Introduction

Acidic soil with aluminum (Al) availability is one of the environmental factors which compromises plant growth in many parts of the world, because in such soil the increase of this metal availability reduces plant development in most cases (Poschenrieder et al., 2008; Kochian et al., 2015). In general, the Brazilian savanna soil, including the rupestrian fields, is acidic, oligotrophic and containing high Al concentrations (Oliveira et al., 2015). However, throughout the world, some plants not only tolerate Al, but also accumulate significant quantities in the shoots and are therefore identified as Al accumulators (Jansen et al., 2002a), for example, *Camellia sinensis* (Theaceae) leaves are used for tea and may accumulate up to 30,000 mg Al kg^{-1} of dry mass (Matsumoto et al., 1976). Many of these Al accumulating plants are found in the Brazilian flora, especially species of the Melastomataceae, Vochysiaceae and Rubiaceae families (Souza et al., 2015), and very little is known about the ecological adaptation traits of these species.

Arboreal species or bushes frequently found in rupestrian fields such as those represented by the Vochysiaceae family, including *Qualea grandiflora*, *Q. parviflora*,

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Vochysia thyrsoidea, *V. haenkeana*, as well as Melastomataceae (*Miconia* spp) and Rubiaceae (*Palicourea rigida*) families, are known to be Al accumulators (Jansen et al., 2002b; Olivares et al., 2010). Some of these species have notable behaviors in terms of adaptation to soluble Al in the soil. For example, contrary to expectations, high Al concentrations in leaves are not associated with low essential nutrient content. While some Al accumulating plants are restricted to oligotrophic soil, others are indifferent to soil fertility (Haridasan, 2000).

The elevated Al content found in shoots of the Al accumulators suggests that these plants have an Al uptake and translocation system which cannot be found in non-accumulating plants (Ma and Hiradate, 2000). Considering the arboreal native species from rupestrian fields, a small number of studies have been conducted. Most of them evaluate the quantitative plant Al accumulation obtained *in situ* and are rarely related to ecological adaptations and to controlled alterations of abiotic factors. Some authors argue that the capacity of Al accumulation is a determining evolving trait in these species to the point that some of them cannot survive without this metal present in the soil (Jansen et al., 2002a). Floristic studies have shown that the Vochysiaceae family has many endemic species in this ecosystem (Shimizu and Yamamoto, 2012) and that in its majority; it has an accumulating trait of more than 1000 mg Al Kg^{-1} in the shoots (Jansen et al., 2002a). These species are spread out in the Espinhaço Mountains in Brazil, and in other areas of the South American region (Shimizu and Yamamoto, 2012). In terms of growth, in general, plants respond positively to soil deacidification, including some native plants of different ecosystems (Haridasan, 2000). Thus, the hypothesis in this study was that minor alterations in soil acidity may interfere in the mineral nutrient and Al accumulation as well as plant development of *Qualea cordata* (Vochysiaceae). The relationship between changes of the acidic soil conditions and its related nutrients and Al levels were verified for the growth of this plant of the Brazilian Cerrado and rupestrian field.

Materials and Methods

Plant material and acidic soil conditions

Qualea cordata plants are an arboreal species, seed propagated, that grow in mesotrophic soil rich in Al and also occur in dystrophic and very acid soils (Haridasan, 2008; França, 2015). The species is found in South America, from Brazil to Bolivia, in savannahs, the Atlantic and cost rainforest (Correa, 1926). The plants grew in a greenhouse from the botanic department of the Federal University of Minas Gerais, Brazil. Seeds and soil used in the experiments were collected in rupestrian fields of the Serra do Cipó National Park, Santana do Riacho, Minas Gerais. The harvested fruits were left in the open at room temperature for 30 days for drying and opened to allow seed removal. After this period, they were kept in the freezer until use.

The soil was collected at 25 cm of depth, in the site of occurrence of this species using a shovel, and the vegetation and organic remains were removed. The soil fertility was analyzed according to Claessen et al. (1997) using the EMBRAPA method in the soil fertility laboratory at the Federal Rural University of Rio de Janeiro.

Seeds were sterilized in 5% hypochlorite for 5 min and then washed 3 times in deionized water. Germination was conducted in plastic gerbox plates covered with 2 germination papers moistened with deionized water, conditioned in germination chamber for 7 days at 30°C and for a 12h photoperiod. After germination, well-

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developed seedlings of the same size were selected for experiments. For growth assays, the seedlings were transplanted to 2 L pots containing the respective soil with or without liming treatment and no fertilizers. The slight variation in soil deacidification was performed by liming ($CaCO_3$), aiming at increasing the soil acidity in one unit only from 4 to 5, which reduces the Al availability by 50%.

Growth evaluation and leaf mineral element content

Plants (n=10) were grown in two different soil acid conditions in the greenhouse for 6 months and irrigated on alternate days with distilled water up to water saturation of the soil. After this period, plants were carefully withdrawn from the pots and the shoot and root lengths were measured. After this, the plants from both soil treatments were separated into stalk, roots and leaves, and then taken to a forced air oven at 60°C until constant mass, to obtain the corresponding dry mass of parts and total biomass. The relation of roots to shoots (R:S) was calculated starting from these values.

The leaf content of the elements iron (Fe), aluminum (Al), magnesium (Mg), calcium (Ca), nitrogen (N) and phosphorus (P) was obtained after dry mass digestion in sulfuric acid and hydrogen peroxide. The P content was determined colorimetrically at 410 nm using ammonium vanadomolybdate and N content, according to the Kjeldahl procedure (Allen, 1989). The Al, Ca and Mg content was obtained through flame atomic absorption spectrometry (FAAS) using Varian, model AA240FS (Mulgrave, Australia).

Chloroplastic pigments and photosynthetic evaluation

For photosynthetic pigment contents, three leaf discs with 1 cm diameter were withdrawn from a totally expanded leaf of five individuals of two soil treatments and submerged in 5 mL of 80% acetone for 48 h at 4°C. These tissues were macerated, centrifuged and the supernatant was used for spectrophotometric determination of chlorophyll *a*, chlorophyll *b* and carotenoids in the following wave-lengths 470, 646 and 663 nm, respectively. The chloroplastic pigment levels were determined as proposed by Lichtethaler and Wellburn (1983).

The photosynthetic parameters were evaluated on a typical day through chlorophyll fluorescence, measured with a modulated fluorescence meter (MINI-PAN, Walz-Germany), and the potential quantic yield values (Fv/Fm) and the effective quantum yield ($\Delta F/Fm'$) was obtained in plants from the acid and less acidic soil. The Fv/Fm from the photosystem II was evaluated in the early morning and the leaves were adapted in the dark for 30 min, being that $Fv/Fm = (Fm - F_0)/Fm$ in which Fm refers to the maximum fluorescence and F_0 to the basal fluorescence. Concerning the $\Delta F/Fm'$ values, these were determined being that $\Delta F/Fm' = (Fm'-F)/Fm'$, in which F is the chlorophyll fluorescence of the sample adapted to the light and Fm' to the maximum fluorescence of samples with saturating pulse.

Statistical analysis

Variance analysis (ANOVA) was carried out to analyze obtained data in the assays. The software used was JMP 5 SAS (Statistical Analysis System 1980, U.S.) and the Tukey test at 5% of probability was employed to compare the obtained means.

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Results

Acidic soil and nutrient levels and plant growth evaluation

The results of fertility soil analysis from the site of occurrence of *Q. cordat*a revealed soil pH values extremely acid with high aluminum content. Also, the nutrient content is low, characterizing the soil as impoverished and dystrophic (*Table 1*).

Table 1. *Fertility analysis of soil collected from the site of occurrence of Qualea cordata Spreng. H2O = pH in water, Ca, Mg and P = calcium, magnesium and phosphorus exchangeable, OM = organic matter, V = base saturation, m = aluminum saturation. Data from 3 composite samples.*

pH	**cmol$_c$ dm^{-3}**		**mg dm^{-3}**	**dag kg^{-1}**	**%**	
H_2O	Ca	Mg	P	OM	V	m
4.0	0.29	0.06	4.91	1.89	6.2	86

The less acidic soil affected negatively and significantly the *Q. cordata* plant growth. The plants grown in more acidic soil presented greater increase in root, leaf and shoot dry weight as well as the total dry weight in comparison to those grown in less acid soil. However, there was no variation in dry weight of the stem or in the root and shoot ratio (R:S) (*Figure 1*). After 180 days of growth, the increment of dry biomass of root, shoot and total biomass of plants from more acidic soil was significantly higher compared to the same values of plants from less acidic soil. However, the R:S ratio was not significant.

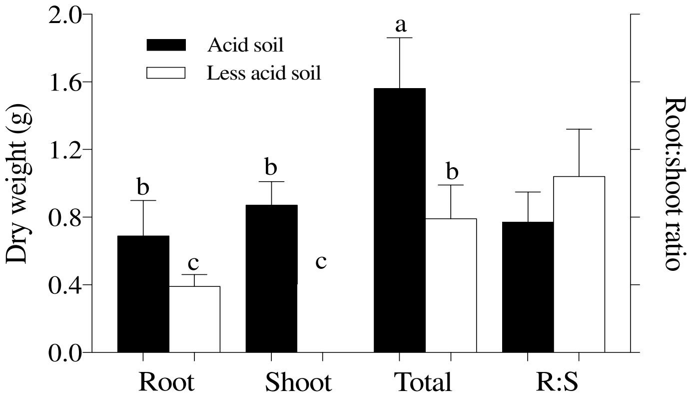

Figure 1. *Root, shoot and total dry weight (g) and root: shoot ratio of Qualea cordata plants after 6 months of growing in more acidic soil and also in less acidic soil. Means followed by the same lowercase letter in the column did not differ significantly by Tukey test at P ≤ 0.05. Means followed by standard deviation with n = 10.*

Regarding the total plant height (*Table 2*), those grown in more acidic soil presented an average apex root length of 43.9cm, while those obtained in the less acidic soil presented 45.0cm. However, the greatest length of roots was found in plants in the less acid soil, and there was a difference in the shoot height of plants in this soil in contrast to those obtained after growth from less acidic soil.

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Table 2. *Root, shoot and total length (cm) of the Qualea cordata plants after 6 months of growing in more acidic soil and also in less acidic soil. Means followed by the same lowercase letter in the column did not differ significantly by Tukey test at $P \leq 0.05$. Means followed by standard deviation with $n = 10$.*

	Root	**Shoot**	**Total**
Acid soil	27.27 ± 1.72 b	16.61 ± 2.95 b	43.89 ± 2.09 b
Less acid soil	32.20 ± 2.89 a	12.79 ± 1.94 c	44.99 ± 3.48 b

Leaf mineral element analysis

In terms of the mineral element content in leaves, the greatest values of N, P, Ca and Mg were found on leaves of plants grown in less acidic soil (*Figure 2*). Contrastingly, the Al leaf content was greater in plants grown in more acidic soil.

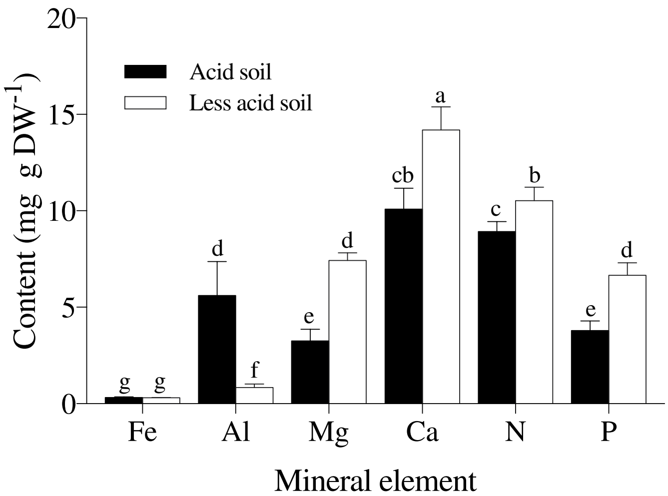

Figure 2. *Content of elements iron (Fe), aluminum (Al), magnesium (Mg), calcium (Ca), nitrogen (N) and phosphorus (P) in the leaves of Qualea cordata after 6 months of growing in more acidic soil and also in less acidic soil. Means and standard deviation with $n = 3$.*

Chloroplastic pigments and photosynthetic evaluation

The total chlorophyll content, distributed among *a* and *b*, was not altered in plants from less acidic soil (*Table 3*), but the carotenoid content and the chlorophyll: carotenoid ratio changed, where the carotenoid content was two times greater in those plants grown in this deacidification soil.

The analysis of fluorescence of chlorophyll *a* (*Figure 3*) indicated discrete lower values of effective quantum yield (ΔF/F'm) and potential (Fv/Fm) quantum yield in the early morning hours in plants from more acidic soil. The Fv/Fm values remained below 0.8 and reduced progressively in the evaluated time between 7 am and 1 pm. However, the ΔF/F'm values diminished drastically at 9 am, raising at 10 am and decreasing again and keeping constant from as of 12 pm. At the same time, the plants cultivated in more

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acidic soil presented values lower that 0.77 for both parameters. The ΔF/F'm values decreased at 9 am and remained below 0.46 during measurements. The variations of these parameters were similar regardless of the soil treatments, but there was a less significant decrease of ΔF/F'm in plants grown in more acidic soil, without statistical differences between soil treatments.

Table 3. *Chloroplastic pigment contents (μg cm-2) in leaves of Qualea cordata plants after 6 months of growing in more acidic soil and also in less acidic soil. Means followed by the same lowercase letter in the column did not differ significantly by Tukey test at P ≤ 0.05 with n = 5. Chl = Chlorophyll; Cx = carotenoids.*

	Chl a	***Chl b***	**Cx**	**Total *Chl***	***Chl*/Cx**
Acid soil	16 a	9 a	3 b	25 a	8.6 a
Less acid soil	21 a	8 a	7 a	29 a	4.0 b

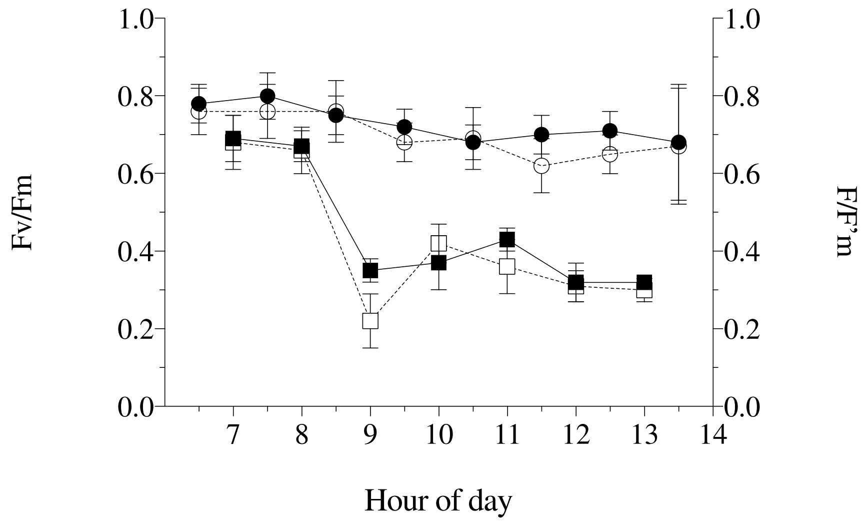

Figure 3. *Potential quantum yield (Fv/Fm) (circles) and effective quantum yield (ΔF/F'm) (squares) in the leaves of Qualea cordata after 6 months of growing in more acidic soil (white symbol) and also in less acidic soil (black symbol). Measures obtained on a clear day from 6:30 am to 1:30 pm. Means and standard deviation with n = 3.*

Discussion

Soil nutrient levels and plant growth evaluation

The soil nutrient analysis at the occurrence site of *Q. cordata* revealed very low values of the base saturation such as the content of P, Ca and Mg, and organic matter as it has been highlighted for rupestrian field soils (Baligar et al., 1995; Oliveira et al., 2015). Contrastingly, elevated values of Al saturation were found, making the ion Al the most predominant cation (Benites et al., 2003). However, the acidity and elevated Al saturation found on site were above those generally found in the Brazilian Cerrado (Baligar et al., 1995). Despite the elevated acidity, excessive Al content and

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impoverished soil that were found, the greatest total biomass accumulation was evident in plants that were grown in this soil, and the shoot was larger in comparison to the roots, that is, a greater investment in structural carbon took place in the shoot. Contrastingly, plants from less acidic soil, there was an equitable partition between shoot and roots. Considering the present chemical soil conditions, the results suggest that the photoassimilated drain in the roots was as strong as of that in the shoots. For *Miconia albicans*, Haridasan (2008) showed that when plants grow in acidic and fertile soil, there was a greater increase in height and biomass, compared to those from acidic and oligotrophic soil, and in alkaline soil, plant growth was significantly less than in those from acidic and impoverished soil. In the present study, there were no nutrients added to the soil, but deacidification was enough to modify the carbon partition. The results also showed a larger carbon displacement for biochemical purposes due to the smaller biomass incorporation of plants in less acidic soil.

There was no difference in the root: shoot (R:S) mass ratio obtained in plants that were grown in less acidic soil, but in relation to the plant length, this condition favored an increase in root size but not in the shoot. In the more acidic soil, a larger mass and a shorter root were indicators of a greater root diameter, and the inversion of these parameters in the less acidic soil suggests thinner roots. The greatest root system length in less acidic soil can be related to alterations in soil nutrient to improve the root uptake system, however, it is also considered that even an Al tolerant plant develops symptoms associated with metal toxicity, such as the thickening and shortening of roots (Silva et al., 2014).

Soil and leaves mineral content

It is known that soil deacidification promotes Al and Fe precipitation which is exchangeable, and reduces P precipitation, increasing labile P fraction (Souza et al., 2007). This was confirmed through the greatest P content found in leaves. A large N content was also observed in plant leaves from less acidic soil; however, the greatest presence of these elements did not induce the greatest growth of shoots or biomass accumulation. The N and P contents found in *Q. cordata* leaves, after growth in both acidic soil conditions, were similar to those found in *Q. grandiflora* and *Q. parviflora* (Nardoto et al., 2006) and other arboreal species in natural field conditions (Delitti et al., 2006). The Ca and Mg contents in leaves from less acidic soil increased significantly, mainly the Mg content with values nearly twice as high and this response had already been previously reported in other native and cultivated plant species of this ecosystem (Furtini Neto, 1999). In *Plantago almogravensis*, Al hyperaccumulator plant, the concentrations of Ca and Mg in the shoot were maintained along the Al gradient (Serrano et al., 2011). In *Q. cordata* the greatest content of N, Ca, Mg and P in leaves, despite not having induced the greatest biomass accumulation in plants, is closely related to the greatest uptake of these nutrients due to soil deacidification, and to the root system length which was approximately 20% higher.

According to Jansen et al. (2002b) the Al content found in leaves of *Q. cordata* (5.6 ± 1.77 mg g^{-1}) from more acidic soil allows this species to be included into the group of Al accumulating plants. However, in plant leaves from the less acidic soil the Al content found was significantly reduced, different than what was found by Haridasan (2000), who said that the increase of oligotrophic soil pH and the higher Ca content in mesotrophic soil did not reduce the Al accumulation in Al accumulating plants. The reduction of Al and biomass accumulation in plants from less acidic soil indicates a

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relation of this metal to the growth of *Q. cordata*. Although these observations refer to the involvement of Al in the cell physiology, there is still no definition of its role in the plant metabolism, even though some authors consider the actions of Al in plants to be beneficial (Haridasan, 2000; Pilon-Smits et al., 2009; Hajiboland et al., 2013). Recently, in *Camellia sinensis*, a hyper Al accumulating plant, the toxic fluorine effect was relieved in the presence of Al (Yang et al., 2016). In rice, it was also found that Al relieves Mn toxicity (Wang et al., 2015). Watanabe et al. (2005) evaluated the beneficial effects of Al in the growth of *Melastoma malabathricum* and when its roots were exposed to 0.5mM of Al, an increase in root growth and Al accumulation in leaves were observed. This only took place in plants grown in complete nutrient solution, evidencing that without nutrients there was no stimulatory effect of Al. Contrastingly, it is expected that soil deacidification may reduce the availability of some micronutrients (Buni, 2014), which can constrain plant growth. In natural acidic soil conditions with higher Al content, Olivares et al. (2010) highlights the positive correlation between Al, Fe and Zn leaf content in 19 Melastomataceae species, some being Al accumulators.

Chloroplastic pigments and photosynthetic evaluation

No changes were observed in the chloroplastic pigment of plants from less acidic soil, except the carotenoid content. Although an increase was expected, the chlorophyll content remained unchanged, since there is a positive correlation to the N content (Verhoeven et al., 1997). Interestingly, despite the less acidic soil plants having accumulated more N, it was not converted to more chlorophyll synthesis that could enhance the photosynthetic capacity. It is probable that the N was conducted for enzyme synthesis for the metabolism of other nutrients and perhaps if the soil organic matter content were higher, the greatest N availability/uptake could have enhanced photosynthetic activity. Haridasan (2008), cultivated *Miconia albicans* and *Vochysia thyrsoidea* in calcareous soil and observed leaf chlorosis. This symptom disappeared in these plants removed to acidic soil whereas in *Q. cordata*, leaf chlorosis was not observed. Also, in *Q. grandiflora*, Al enters the chloroplasts without apparent damage to this organelle (Andrade et al., 2011). In relation to the carotenoid content, Franco et al., (2007) suggested that zeaxanthin plays an important role in the dissipation of non-photochemical energy in trees at moments of higher irradiance. Thus, the highest carotenoid content found in plants from less acidic soil suggests that the change in soil acidity generated the need for more photoprotective capacity, evidenced by the increase in *Chl*:Cx ratio.

Chlorophyll fluorescence data indicated chronic photoinhibition in *Q. cordata* leaves in the evaluated time period. The performance of the photosynthetic apparatus observed by Fv/Fm and ΔF/F'm did not change in plants from less acidic soil, indicating that lower carbon incorporation would result in greater damage to photosystem II, as often reported in plants under stress due to the lack or excess of boron (Pinho et al., 2010), nitrogen (Tripodi and Sievering, 2010) or copper (Padua et al., 2010). The slight photoinhibition observed since dawn (Fv/Fm < 0.8) became more pronounced with the increase of irradiance. Franco et al. (2007), demonstrated similar behavior in *Schefflera macrocarpa* and *Ouratea hexasperma* and suggested that increased vulnerability to photoinhibition in the morning was reversed by the cycle activity of the xanthophylls, leading to an improvement in the photochemical process. Thus, in this plant growth stage, the results pointed to the failure of adaptation to higher photosynthetic rates, as constitutive levels of chlorophyll are maintained, and there was greater biomass

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incorporation even with an increased nutrient uptake. However, the increase of carotenoid level indicated the need for photoprotection and without this mechanism the photoinhibition could be more severe in plants from less acidic soil conditions.

Conclusions

Our data support the ecological adaptation of *Q. cordata* growth in impoverished rupestrian field soils and can also explain the distribution pattern of the species in extremely acidic soil with high Al content. This research revealed that such species is an Al accumulating plant. The slight variation of soil deacidification in the occurrence areas of this species, and other plants with similar ecological chemical soil adaptations, can induce a constraint in the reintroduction process, once it leads to reduced initial growth and its possible plant establishment. Despite the results obtained in controlled conditions, the resilience of this species and also other Al accumulator plants should be revealed in field conditions, like other environmental constraints that compromise the growth of native plants.

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APPENDIX

Appendix 1. *Dry weight mass (DW) of root, shoot and total biomass and root: shoot ratio (R:S) of the Qualea cordata plants cultivated for 6 months in two acid soil conditions (pH:4 and pH:5). N=10, SD: standard deviation.*

	Acid soil	**Less acid soil**
	DW(SD)	DW(SD)
Root	0.69(0.20)	0.39(0.07)
Shoot	0.87(0.14)	0.40(0.07)
Total	1.56(0.30)	0.79(0.20)
R:S	0.77(0.77)	1.04(0.28)

Appendix 2. *Mineral element content (mg g DW^{1}) in leaves of **Qualea cordata** plants cultivated for 6 months in two acid soil (pH:4 and pH:5) conditions, N=3.*

	Acid soil			**Less acid soil**		
Fe	0.28	0.32	0.36	0.3	0.28	0.32
Al	3.83	5.60	7.37	0.66	0.88	1.0
Mg	2.64	3.25	3.86	7.04	7.43	7.82
Ca	11.18	10.09	9.0	15.4	14.19	13.0
N	8.44	8.92	9.44	9.82	10.52	11.22
P	3.3	3.80	4.29	5.99	6.65	7.31

Appendix 3. *Potential quantum yield values (Fv/Fm) and effective quantum yield values (ΔF/F'm) obtained in leaves of **Qualea cordata** plants cultivated for 6 months in two acid soil (pH:4 and pH:5) conditions. N=3, SD: standard deviation.*

	Acid soil		**Less acid soil**	
	Fv/Fm	F/F'm	Fv/Fm	F/F'm
6:30 am	0.78(0.05)	-	0.76(0.06)	-
7:00 am	-	0.68(0.07)	-	0.69(0.06)
7:30 am	0.8(0.06)	-	0.76(0.07)	-
8:00 am	-	0.66(0.06)	-	0.67(0.04)
8:30 am	0.75(0.05)	-	0.76(0.08)	-
9:00 am	-	0.22(0.07)	-	0.35(0.03)
9:30 am	0.72(0.05)	-	0.68(0.05)	-
10:00 am	-	0.42(0.05)	-	0.37(0.07)
10:30 am	0.68(0.05)	-	0.69(0.08)	-
11:00 am	-	0.36(0.07)	-	0.43(0.03)
11:30 am	0.70(0.05)	-	0.62(0.07)	-
12:00 am	-	0.31(0.04)	-	0.32(0.05)
12:30 pm	0.71(0.05)	-	0.65(0.05)	-
13:00 pm	-	0.3(0.03)	-	0.32(0.01)
13:30 pm	0.68(0.15)	-	0.67(0.15)	-

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THE COMPARISON OF TOTAL PHENOLIC CONTENT, ANTIOXIDANT CAPACITY AND MOLECULAR ANALYSIS OF SOME SELECTED TURKISH APPLE (*MALUS SPP.*) GENOTYPES

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Abstract. In the present study, eight apple genotypes were analyzed using internal cpDNA trnL intron sequence. Furthermore, these genotypes species were evaluated for their antioxidant activity, total phenolic and flavonoid contents. Genotypes in question show quite a variation in biochemical characteristics and molecular systematic analysis. One of the genotypes “Helesan” was the best graded genotype based on the high content of the total phenolic (12,21±1,25) and total flavonoid (7,98±0,26) content. As a result of the molecular analysis, which is one of the main aims of the study, genotypes are comprised of three main clades with robust bootstrap (95) value. It was concluded that these genotypes could be useful to improve genetic diversity among genotypes for breeding.
Keywords: *antioxidant; Malus sp.; cpDNA trnL; molecular systematic*

Introduction

Hundreds of clinical trials and epidemiological studies have been carried out to investigate the effect of diet on health. Although not all studies show a link between vegetable and fruit and health, in a major part of them, positive correlation is observed between vegetable, fruit consumption and health and reduced risk of some chronic diseases such as cancer, cardiovascular diseases (Arts and Hollman, 2005; Boyer and Liu, 2003; Lampe, 1999). This association partially may be due to the antioxidant compounds present in fruits showing protective effect on the cell against oxidative effect caused by reactive oxygen species (ROS) and reactive N species (RNS) (Arts and Hollman, 2005; Henríquez et al., 2010; Scalbert et al., 2005). Therefore, it is very important to consume foods rich in antioxidants. These compounds may reduce oxidative stress through a number of mechanisms (Kalinowska et al., 2014). The total antioxidant capacity of fruit extracts was measured by using spectrophotometric approaches with DPPH (2,2-diphenyl-1-picrylhydrazyl) (Rivero-Pérez et al., 2007). DPPH method is based on the electron transfer from an antioxidant to a free radical (Duarte-Almeida et al., 2006).

Apple (Malus × domestica Borkh.) is among the commonly consumed fruits. In Turkey, apple production has reached to 2.57 million ton/year (Bugem). Apple makes up a significant part of human diet as they have sugars, acids and a number of biologically active compounds such as phenolic and flavonoid compound (Boyer and Liu, 2003; Lampe, 1999; Wu et al., 2007). There is a strong link between phenolic content of apple and antioxidant activity. The main phenolics acid found in apple is chlorogenic acid and this compound has the ability to scavenge free radicals (Panzella et al., 2013). The concentration of these phytochemicals including phenolics and flavonoids compounds greatly depends on the apple genotype and the maturity stage of the apples and is closely related to their nutritional and sensory properties (Wu et al., 2007).

Though there are over 7,000 apple genotypes known across the globe, nowadays the world's production is based on a limited number of genotypes (Hokanson et al., 2001; Patzak et al., 2012). Therefore, A genetic characterization of the apple genotypes plays a key role in breeding programs, patent protection and nursery control (Goulão and Oliveira, 2001; Smolik et al., 2004). The recent advances in DNA technology have provided several methods which may be employed for the characterization of apple germplasm collections (Patzak et al., 2012). The trnL-F region, consisted of the trnL intron and trnL-F spacer, has been among the commonly used chloroplast markers for phylogenetic analyses in plants. The trnL-F region has been found to be useful in molecular phylogenetic studies of a broad range of plant groups (Pirie et al., 2007; Quandt et al., 2004).

Therefore, the aim of this study is: (a) to determine the total phenolic and flavonoid contents and the antioxidant activity of the eight native apple genotypes, (b) to assess the phylogenetic relationship between genotypes by alignments of DNA sequences from *cpDNA trnL-F* region.

Material and methods

Apple genotypes

Eight apple genotypes (Benekli, Helesan, Hese, Ovacin, Seva Zer, Sıvi Şirin, Sıvi Tırş, Sohrık) from *Malus domestica* otor were analyzed in this study. The name of the apple genotypes is given in *Table 1*.

Table 1. *Total phenolic and total flavonoid contents of peel for different apple genotypes*

Apple genotypes	TPC (mg GAE/g)	TFC (mg RE/g)
1. Benekli	3,75±1,64[cd]	4,49±0,13[e]
2. Helesan	12,21±1,25[a]	7,98±0,26[a]
3. Hese	5,22±0,83[bc]	5,21±0,22[bc]
4. Ovacin	4,23±0,56[bc]	4,79±0,16[de]
5. Seva Zer	2,57±0,41[d]	4,02±0,11[f]
6. Sıvi Şirin	4,67±0,21[bc]	5,01±0,38[cd]
7. Sıvi Tırş	4,87±0,20[bc]	5,37±0,19[bc]
8. Sohrık	5,78±0,31[b]	5,47±0,17[b]

TPC – total phenolic content; TFC – total flavonoid content; GAE – gallic acid equivalents; RE – rutin equivalent. The results were expressed as mean ± standard deviation. Means sharing the same letter in a column are not significantly different by Duncan's multiple range test at P ≤ 0.01

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All genotypes were identified by specimen. The apples were collected during harvest seasons (June-August, 2013-2015). Fresh peel was manually separated from the pulp and seeds with the help of pens and forceps and then was dried until reaching a constant weight. The fresh peel was powdered with mortar and pestle by methanol (80 %) and was shaken at dark for a night. The extracts were evaporated by the rotary evaporator and solutions of 1 mg/ml concentration were prepared. For phylogenetic analysis, the leaves belonging to each apple genotypes were protected in silica gel and the samples were homogenized with liquid nitrogen and stored at -20°C.

Preparation of apple genotypes

The powdered peels from eight apple genotypes were weighted (1.0 gr) and mixed with methanol (80 %). The samples were homogenized with the homogenizer for 2 minutes and subjected to the ultrasound for five minutes. The extracts were shaken on an orbital shaker at midnight and evaporated to dryness. The concentration of all crude extracts was adjusted to the same value through addition of 80 % methanol.

Total phenol contents

Total phenolic contents (TPC) of powdered apple peels were performed by methods involving Folin–Ciocalteu reagent and gallic acid as standard (Slinkard and Singleton, 1977). Extract solution (0.1 ml) containing 1,000 mg extract was taken in a tube, and 1 ml Folin–Ciocalteu reagent was added and the flask was shaken thoroughly. After 3 min, 1 ml of solution of 6 % Na_2CO_3 was added and the mixture was allowed to stand for 1 h with intermittent shaking. Absorbance was measured at 760 nm with a Shimadzu UV mini 1240 spectrophotometer (Japan). The same procedure was repeated to all standard gallic acid solutions.

Total flavonoid content

The total flavonoid content was estimated using aluminum chloride colorimetric assay (Zhishen et al., 1999; Zou et al., 2004). The 0.5 ml of test samples solution in methanol were mixed with 2 ml of distilled water and 150 µl of 5 % sodium nitrate. After 6 min, 150 µl of 10 % aluminum chloride and 2ml of 1 M sodium hydroxide were added and left at room temperature for 15 min. The absorbance of mixtures was measured at 510 nm. Rutin was used as a standard to determine flavonoid contents of the grape seed extracts.

DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed in accordance with (Villano et al., 2006). Concentrations were adjusted to 1, 5 and 10 mg/ml respectively. Briefly, 1 ml of sample was added to the freshly prepared 4 ml of 0.01 mM DPPH solution (dissolved in methanol), incubated for 15 min in dark conditions and measured at 517 nm (*Eq. 1*).

$$\text{DPPH activity}\ (\%\ \text{incubation}) = \left(A_C - A_1\right) / A_C \times 100 \qquad \text{(Eq. 1)}$$

(A_C: Control Absorbance, A_1: Sample Absorbance)

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Statistical analysis

Results obtained were reported as mean ± SD of triplicate measurements. Significance differences for multiple comparisons were determined by one way analysis of variance (ANOVA) followed by Duncan test with $\alpha = 0.01$ using SPSS (version 20).

DNA isolation, PCR condition and phylogenetic analysis

DNA was extracted from leaf tissues of genotypes according to CTAB (cetyl trimethylammonium bromide) method (Doyle, 1987). DNA quality was checked on 1 % agarose gel and then quantification was measured by using the Nanodrop (Thermo). To achieve the amplification of trnL-F region of chloroplast (cp) genome, PCR was performed in 25 µl volume. That volume was occurred from 3 µl (100 ng) DNA template 2,5 µl 10X taq buffer, 3 µl $MgCI_2$, 1 µl forward, reverse primers and 10 mM dNTP mix, 0,25 µl Taq polymerase and finally 13 µl ddH_2O. PCR products were separated by gel electrophoresis on 1.5 % agarose gels, containing ethidium bromide, and photographed under UV light in a gel doc system. PCR products of trnL-F region were sequenced by Iontek Company, Istanbul, Turkey.

Alignment and phylogenetic analysis

The sequences obtained were blasted (basic local alignment search tool) in NCBI (National Center for Biotechnology Information) database and percentage homology scores were assessed to identify apple genotypes. Phylogenetic trees were done with MEGA version 6 using a neighbor-joining algorithm with bootstrap analyses for 1,000 replicates.

Results and discussion

Antioxidant properties

Total phenolics and flavonoids

The total phenolic content (TPC) and the total flavonoid content of the apple peel of all genotypes, quantified as gallic acid equivalent and rutin respectively, are shown in *Table 1*. The TPC varied considerably depending on the genotype. Of all the apple genotypes, the highest TPC was observed in Helesan, whereas Seva Zer had the lowest TPC. In addition, the highest total phenolic content (TFC) was found in Helesan followed by Sohrık. As regards total flavonoid contents, they changed between 4,02 and 7,98. There was a significant difference at $p < 0.01$ between all samples for the total phenolic and flavonoid contents.

These results are incongruent with (Vieira et al., 2009; Manzoor et al., 2012) but in line with the results of (Wolfe et al., 2003; Faramarzi et al., 2014; Leahu and Ropciuc, 2013).

According to the results of previous studies (Awad and de Jager, 2000; Duda-Chodak et al., 2011; Escarpa and Gonzalez, 1998; Łata et al., 2009), it can be noted that some apple genotypes have higher TPC than others (Kalinowska et al., 2014). For example, the peel of ‘Red Rome’, ‘Idared’, ‘Fiesta’, ‘Fuji’, ‘Gloster’, ‘McIntosh’ and ‘Pilot’ contains higher amount of chlorogenic acid in comparison to other varieties, whereas ‘Elan’, ‘Elstar’ and ‘Jonamac’ show the lowest content of chlorogenic acid. ‘Red

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Rome', 'Prima', 'Pilot' and 'Elstar' possess high amount of catechin and epicatechin in peel. 'Starking Delicious', 'Gloster', 'Golden Delicious', 'Granny Smith', 'Idared' and 'Monroe' possess high content of phloridzin. While the peel of 'McIntosh' and 'Reineta' are abundant in procyanidin B1, 'Elstar' and 'Idared' in procyanidin B2, 'Gloster', 'Idared', 'Lobo' and 'Elstar' are rich in procyanidin C1. The composition of the peel dependent on apple genotype differs significantly. However, most of the phloridzin, procyanidines B2 and C1, catechin and epicatechins are present in apple fruit (Carbone et al., 2011). The differences among TPCs can be attributed to the presence of the mixture of different cyanidin glycosides (Tsao et al., 2005; Wolfe et al., 2003) or to the biosynthesis pathway of phenolic compounds (Treutter, 2001; Wang et al., 2013). Fruit maturation also plays an important role on the concentration of the phenolic compounds in apple peel (Treutter, 2001). Moreover, since all eight apple genotypes tested in the present study were grown in the same region with similar ecological conditions, the variation in total phenolics and flavonoids demonstrates that the genetic variability may have given rise to the differences in the biosynthesis of phenolic secondary metabolites.

DPPH radical scavenging activity

The DPPH free radical is a constant free radical which has been utilized as a tool for determination of free radical scavenging activity of antioxidants (Acharya et al., 2012; Fenglin et al., 2004; Oyaizu, 1986). The results are presented in *Table 2*. According to DPPH scavenging activity assay results, the highest scavenging capacity was found in Hese extract followed by Sıvi Şirin of 5 mg/ml concentration with a clear difference, and for all the genotypes studied these values ranged between 79,62 % and 77,43 %.

Table 2. *Free radical scavenging activity (Percentage) of 8 different apple genotypes in the concentrations of 5 mg/ml by DPPH reduction*

Genotypes	Inhibition (%)
Benekli	77,43
Helesan	74,92
Hese	79,62
Ovacin	75,86
Seva Zer	77,74
Sıvi Şirin	78,06
Sıvi Tırş	77,43
Sohrık	77,74

Our findings are in disagreement with some research results (Jelodarian et al., 2012; Yoshizawa et al., 2005), but consistent with some others (Manzoor et al., 2012; Sownthariya and Shanthi, 2015).

It has been noted that free radical scavenging activity of the plant based food (fruits and vegetables) extracts principally arise from phenolic compounds, which are largely distributed in the epidermal tissue (Cheng et al., 2006; D'Abrosca et al., 2007). Furthermore, the method used for determination of antioxidant activity has

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various effects on results. It should be mentioned that antioxidant activity can be affected by the solvent type. As different extraction solvents differ greatly in terms of polarity, their extracting abilities for different compounds are different (Kong et al., 2012; Li et al., 2014; Liu et al., 2009; Razali et al., 2012). For example, Li et al. (2014) reported that DPPH values of 10 crab apples (Malus wild species) ethanol extracts were found 6 times higher than ethyl acetate extracts on average. Apart from the solvent type used, the extraction method used is another important parameter. He et al. (2015) used three different extractions methods (microwave-assisted extraction, ultrasound-assisted extraction and water bath extraction) for the extraction of apple polyphenols and concluded that microwave-assisted extraction was much better than the other two methods.

Molecular phylogeny of apple genotypes using the chloroplast trnL-F DNA sequences

Chloroplast DNA (cpDNA) is a robust tool for inferring the interspecific relationship between the plant taxon. To study the genetic relationship of the interspecific genotypes between trnL (UAA) 3'exon and trnF (GAA) gene is known quite informative (Ashworth, 2017; Taberlet et al., 1991). In this study, a molecular phylogenetic analysis of eight apple genotypes commonly consumed in Turkey was performed by using trnL-F cpDNA sequences.

trnL-F cpDNA sequences are usually used for molecular classification plant cultivars, genotypes, species and interspecies analyses (Ashworth, 2017). In our study, ML and NJ phylogenetic analysis of trnL-F sequences showed almost the same profile but only NJ tree with *Mespilus germenica* out group is presented here (*Figure 1*).

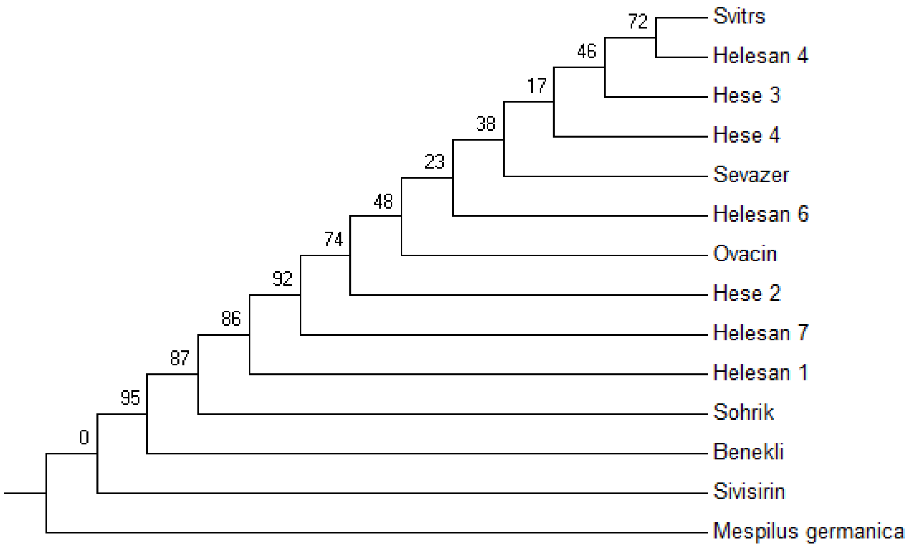

Figure 1. *Phylogenetic analysis of apple genotypes based on trnL-F chloroplast region with Neighbour-Joining method (numbers on branches show the bootstrap value)*

Based on the NJ tree, genotypes are comprised of three main clades with a robust bootstrap (95) value. The widest of these three clades comprised the Sivitırs, Helesan 4, Hese 3, Hese 4, Sevazer, Helesan 6, Ovacin, Hese 2, Helesan 7, Helesan 1 and Sohrik genotypes. The second clade comprised only Benekli genotype and Sivisirin constituted the obviously separated clade (*Figure 1*). In the first clade, it was found that all replicate

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genotypes fell within the same group. Thus, genetic relationships of the apple genotypes based on trnL-F region were estimated to be quite effective. There are many phylogenetic studies that found that trnL-F cpDNA region was quite effective for separating plant genotypes (Ashworth, 2017; Zhang et al., 2014; Zhang et al., 2016). In the present study, polymorphism of trnL-F analysis was found to be more informative. It produced fairly clear dendrogram that explains all apple genotypes according to morphological character and aromatic properties. To support this point in our analysis, Sivisirin apple genotype, which has different aromatic feature (it is known so sweet), comprised an evidently separated clade (*Figure 1*). Furthermore, this genotype has the highest inhibition rate (78 %) in DPPH analysis (*Table 2*). Sivisirin also showed a different value according to other genotypes in TPC and TFC analysis (*Table 1*).

Conclusion

This study was carried out to determine the biochemical and molecular relationships of eight apple genotypes that were collected from different regions of Turkey. The relevant genotypes show quite variation in biochemical characteristics and molecular systematic analysis. "Helesan", one of the genotypes, was the best graded genotype based on the high content of the total phenolic (12,21±1,25) and total flavonoid (7,98±0,26) content. As a result of the molecular analysis, which is granted as one of the main points of the study, genotypes are comprised of three main clades with a robust bootstrap (95) value. The widest of these three clades comprised the Sivitırs, Helesan 4, Hese 3, Hese 4, Sevazer, Helesan 6, Ovacin, Hese 2, Helesan 7, Helesan 1 and Sohrik genotypes. The second clade comprised only the Benekli genotype and Sivisirin comprised the obviously separated clade. Consequently, it is expected that this study will be a useful step for the molecular biotechnology breeding in horticulture agriculture. These local apple genotypes can also be assessed as a quite important source of genes for apple breeding program and for the production of value added apple genotypes. Hence, further studies are recommended on local and ancient genotypes to identify their biological potentials.

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TOXICITY ASSESSMENT OF *ANABAENA SP.* FOLLOWING EXPOSURE TO COPPER OXIDE NANOPARTICLES AND SODIUM CHLORIDE

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Abstract. The widespread use of nanomaterials in recent years has caused rising pollution in surface and waste waters. It may be seriously affecting aquatic life such as cyanobacteria and thus, there is a compelling need to evaluate the potential effects of these substances in aquatic ecosystems. Copper oxide is one of the most common nanoparticles (CuO-NPs) used today. In this study, the responses of *Anabaena sp.* to CuO-NPs, salt stress and their combined effects (CuO-NPs+NaCl) were investigated. After 72 h exposure, MDA (malondialdehyde), proline, protein content, soluble sugar, total phenols and the activities of antioxidative enzymes such as peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and polyphenol oxidase (PPO) were measured using spectrophotometry. Scanning electron microscopy (SEM) was used to image the results of exposure of cyanobacterial cells to nanoparticles. The effective concentrations (EC10, EC50, EC90), no observed effect concentration (NOEC) and percent inhibition (I %) was also calculated after 24, 48 and 72 hours. EC and NOEC data for CuO-NPs treatments were considerably higher than were those for NaCl treatments. CuO-NPs had a significant impact on the shape and morphology of *Anabaena sp.* cells and resulted in their swelling and enlargement. Modulations in enzymatic and non-enzymatic antioxidants were clearly evident in *Anabaena sp.* exposed to salt and CuO-NPs stress. Lipid peroxidation, measured in terms of MDA levels, increased with CuO-NPs and CuO-NPs+NaCl stress. Flavonoid and proline contents were also increased by CuO-NPs stress. Similarly, increases in POD and CAT activities were more pronounced in the CuO-NPs+NaCl treatments. On the other hand, APX activity enhancement was strongest in *Anabaena sp.* exposed to CuO-NPs.
Keywords: *antioxidant activity, aquatic ecosystems pollution, cyanobacteria, CuO-NPs, salinity, two combinational stress*

Introduction

Nanomaterials, products of nanotechnology, are an important part of numerous industrial and medical products and their use has increased dramatically in the past decade (Peralta-Videa et al., 2011). Being so widely used, it is likely that they will increasingly contaminate aquatic ecosystems (Klaine et al., 2008). Despite this obvious eventuality, the toxic ecological effects of nanomaterials have nevertheless not been easily evaluable; the mechanisms of toxicity of nanoparticles are not understood (Dhawan et al., 2009). The key physicochemical features of nanomaterials associated with toxicity which have been identified by toxicological researchers to date include factors such as aggregation, solubility, size, shape, elemental

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composition, contact area, porosity, surface ionic charge, and hydrodynamic diameter (Griffitt et al., 2008; Johnston et al., 2010, Peralta-Videa et al., 2011).

Biological responses of organisms are very much dependent on the physicochemical properties of nanoparticles. For example, nanoparticles are highly mobile in water and thus they can easily pollute aquatic ecosystems (Oukarroum et al., 2012). Nanotoxicity, as one of the most important aspects of nanotechnology, is induced by nanomaterials whose safety and toxicity characteristics are not clearly defined; and therefore must be determined to ensure public safety. In addition, high concentrations of salts, especially sodium chloride, are among the most important abiotic factors which can affect the survival of living organisms, including aquatic organisms such as cyanobacteria (Denizet al., 2011). The presence of toxic ions, mainly Na^+, creates distress due to osmotic potential and their effect on the uptake of inorganic nutrients (Deniz et al., 2011). This leads directly to inhibitory effects on growth, oxidative stress and finally death. It has been shown that high salinity inhibits photosynthesis, resulting in a decrease in the carbon pool of freshwater cyanobacteria (Srivastava et al., 2008) and lowering of the synthesis of compatible solutes (Ferjani et al., 2003). Furthermore, although nano metals and metal-oxides (Haulik et al., 2014) and salinity have harmful effects on cyanobacteria and plants individually, their effect cannot be directly extrapolated when two or more combinational stresses are induced (Suzuki et al., 2014). Algal cells, in contrast to human and animal cells, possess a cellulose cell wall, which acts as a barrier to particle uptake, prohibiting uptake of nanoparticles larger than 20 nm in size. Nevertheless, nanoparticles are capable of inducing pore formation, which may result in uptake of larger-sized particles (Navarro et al., 2008). Cyanobacteria are major organisms in aquatic productivity and significantly contribute to the availability of nitrogen in agriculture (Singh, 1961) and the evolution of oxygen-dependent respiration. *Anabaena*, as one of the significant cyanobacteria, is not only widely distributed across a wide range of salinities (Srivastava et al., 2011), able to cope with high osmotic potential by enhanced osmolyte synthesis (Hagemann, 2011), but also a preferred candidate for dinitrogen fixation (Singh, 1961). On the other hand, phytoplanktons are used as an early and direct warning signal because of their rapid response to stresses (Del Arco et al., 2014). However, not many studies have previouslybeen conducted on the combined effect of salt and CuO-NPs in cyanobacteria in general and *Anabaena* in particular, although their exposition to many kinds of stresses, individually, has been reported previously (Rai et al., 2013).

In the present study, the impacts of combined effect of CuO-NPs and salt stress were investigated on antioxidant activity and growth inhibition of *Anabaena sp.* collected from Guilan wetlands.

Materials and methods

Nanoparticles

CuO-NPs produced by US Research Nanomaterials Company, were used. The size of used CuO nanoparticles was 40 nm. A 2000 mg/l stock solution was used for preparing appropriate concentrations (*Table 1* and *Fig. 1*).

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Table 1. *CuO nanoparticle characteristics*

Purity	99%
Color	Black
Particle size	40nm
Specific area	20m2/g~
Morphology	nearly spherical
Bulk Density	0.79g/cm3
True Density	6.4g/m3

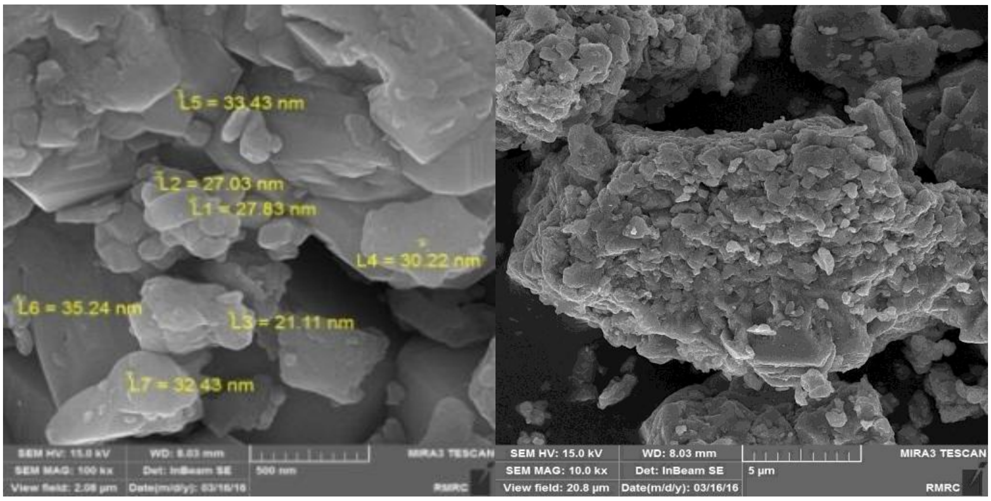

Figure 1. *SEM of CuO-NPs*

Release of Cu^{2+} From CuO-NPs

Stocks of nanoparticles at concentrations of 5, 10, 20 and 25 ppm were prepared with distilled water and the particles dispersed by sonication (Misonix Sonicator 3000, Iran) for 60 minutes. The pH was adjusted to 8.5 using NaOH. After 24 hours, 10 mL of the shaken up solution was taken from each flask, centrifuged 20 minutes at 4500 rpm and filtered through a 150 nm filter membrane. The content of Cu^{2+} was measured by atomic absorption flame spectrometer (Varian Spectra AA 220FS Varian Spectra Co. Australia).

Isolation of Anabaena sp. and preparation of culture medium

Cyanobacteria *Anabaena sp.* were collected from Guilan wetlands and isolated by repeated plating on solid Zander culture medium (Miller, et al., 1978). The pH of the culture medium was adjusted to 6.8, and the culture temperature was maintained at 24 ± 2 ˚C with aphoto period of 12 hours light/12 hours darkness. Light intensity was 60 μmol photon $m^{-2}S^{-1}$. Culture medium was sterilized by autoclaving at 121°C for 15 min and then refrigerated at 6°C.

Preparation of main treatments

The main stock of nanoparticles was prepared by 60 minutes ultrasonic treatment. Six treatments and one control with three replicates for each treatment and control were

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prepared. The concentrations of nanoparticles used in the experiments were 0, 5, 10, 15, 20 and 25 mg/l. 4×10^4 cells from the main stock of *Anabaena sp.* were added to 40 ml of the treatments by dilution of primary algal stock. This step was performed under completely sterile conditions. The test tubes were then stored at 24±2°C which was adjusted by a thermostat. All samples were maintained at constant light periodic condition (12 hours darkness and 12 hours light) for the 72 hours of the experiment.

Determination of growth rates in cyanobacteria

Growth inhibitory effects were studied according to the OECD201 method (Handy et al., 2012). At 24, 48 and 72 hours after the start of the experiment, aliquots of the solution were removed from the test tubes using Pasteur pipettes and the cells present were counted in a Neubauer chamber under an optical microscope. After counting and recording the data, the average number of cells in the top and bottom squares was calculated and then the number of cells was calculated using the following formula:

$$\text{Cell density per ml} = \text{average cells counted in the large square} \times 10^4 \times \text{dilution factor}$$

In each experiment, percent inhibition values were calculated using spectrophotometric data compared with the growth in control systems. EC values were calculated using linear regression analysis of transformed chemical concentration as natural logarithm data versus percent inhibition.

Experimental design and stress application

The EC_{30}doses of single and combined salt and CuO-NPs treatments were determined using the plate colony count method (Rai and Raizada., 1985). A 0.052 M solution of NaCl was used for salt treatment. In addition, 12.58 mg/L of CuO-NPs was used. For the NaCl+CuO-NPs study, the two doses were applied simultaneously. Biochemical evaluations were determined similarly 72 h after treatment. All experiments were done in triplicate.

Antioxidant assays

Assay for total phenolics

Measurement of total phenol was performed by method of Gao et al. (2000), using Folin- Ciocalteu reagent and a standard of gallic acid. For this purpose, 100 µl of extract (2 mg/ ml) was poured into a glass tube. Then, 200 µl Folin solvent and 2 ml of distilled water were added to the extract. 1 ml of sodium carbonate (21%) was added after 9 minutes to the glass tube in darkness. Finally, the samples were stored at room temperature in the dark for an hour. Blanks were prepared with 100 ml of solvent, instead of the extract. Absorptions were read at 765 nm. The total phenolic content was determined as gallic acid equivalents (mg GAE/g extract).

Assay for total flavonoids

Total flavonoid content was determined using a spectrophotometer according to Arvouet-Grand *et al*. (1994). 1 ml of extract solution with 2 mg/ml concentration was mixed with 1 ml of 2% aluminum trichloride ($AlCl_3$) methanolic solution. Absorbance of

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the reaction mixtures were measured at 415 nm against a blank after 10 min. Quercetin (QE) was employed as a standard reference and the total flavonoids content of the extracts was expressed as µg quercetin equivalents per gram of extract (µg QE/g extract).

Extraction and measurement of proline

Proline content in the samples was measured using the method of Bates et al. (1973). For this purpose, 1 g fresh biomass of Cyanobacteria was harvested and their cell walls were broken using 4 ml of 3% sulfosalicylic acid and sonication for 10 minutes (5 pulse seconds and 5 seconds rest). The resulting solution was centrifuged for 20 minutes at a speed of 13,000 rpm and immediately transferred to an ice bath. Finally, 4 ml of toluene was added to the reaction solution and absorbance was measured at 520 nm. Proline content in micrograms per gram fresh weight was calculated.

Total soluble protein

The measurement of total protein was conducted using 2 mg/ml extracts and Coomassie Brilliant Blue G-250 staining solution - in 95% ethanol and 85% ortho phosphoric acid (Bradford, 1976) using bovine serum albumin as a standard.

Lipid peroxidation content assay

Lipid peroxidation was measured in terms of the total content of 2-thiobarbituric acid (TBA)-reactive substances and expressed as equivalent of MDA (malondialdehyde), extinction coefficient 155 mM^{-1}, using the method of Heath and Packer (1968).

Estimation of enzymatic antioxidants

Measuring the activity of catalase (CAT)

The method of Aebi (1984) was used for investigation of catalase activities. For this purpose, 2.5 ml of 0.05 M phosphate buffer was mixed with 0.2 ml of 3% hydrogen peroxide and 0.2 ml enzyme extract. Absorbance was read at 240 nm for 2 minutes. The enzymatic activity of the enzyme was reported as units/mg of protein.

Measuring the activity of ascorbate peroxidase (APX)

APX activity was determined at an absorbance of 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) for 1 min in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, and 200 µl of enzyme extract (Nakano and Asada., 1981)

Measuring the activity of polyphenol oxidase (PPO)

Activity of polyphenol oxidase was assayed according to the method of Grigori (1993). 3 ml reaction solutions containing 2.5 ml phosphate buffer 50 mM (pH =7.2), 0.2 ml Pyrogallol 0.02 M and 300 Micro liters of enzyme extract were used. Changes in absorption were read at 430 nm for 2 minutes. The enzyme activity was calculated based on units of enzyme in 1 gram of fresh weight with extinction coefficient of 0.001 mM^{-1}/cm^{-1}.

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Measuring the activity of Guaiacol peroxidase (POD)

Kinetic activity of Guaiacol peroxidase was determined according to Kalir et al. (1984). First, 1 ml of the reaction solution containing 475 µl Guaiacol 45 mM, 475 µl hydrogen peroxide 100 mM and 50 µl enzyme supernatant was prepared. Changes in absorption due to Guaiacol oxidation were read by spectrophotometer at 470 nm for 2 minutes. An extinction coefficient of 26.6 mM^{-1}/cm^{-1} was used in the calculation of the enzyme activity. Peroxidase enzyme activity was calculated in Unit/gr FW. Each enzymatic unit is the amount of enzyme which increases 0.01% of the absorption at 470 nm per minute.

Electronic images

Scanning electron microscopy (SE-SEM, MIRA3 TESCAN) was used to investigate the effects of nanoparticles on the shape and size of cells in microscopic tissues of *Anabaena sp.* and to image the surfaces. Imaging of the surfaces of control and NP-treated cells was performed on cells exposed to the 10.93 mg/L NP concentration, which had affected 30% of the cells (EC_{30} = 10.93 mg/L).

Statistical analysis

Each treatment was replicated three times; the results were reported as the average of three parallel determinations of the mixture of three replicated samples. All statistical analyses were carried out using SPSS 16.0. One Way ANOVA with Duncan's test was used to determine significant changes in the results ($p < 0.05$).

Results

Release of Cu^{2+}

Suspensions of 0, 5, 10, 20 and 25 mg/l Cu^{2+} nanoparticles caused the release of 0, 0.017, 0.116, 0.249 and 0.310 mg/l of Cu^{2+} respectively, as assayed by atomic absorption flame spectrometry. After 24 hours, less than 0.31 mg/l Cu^{2+} was detected in our experiments (*Fig. 2*).

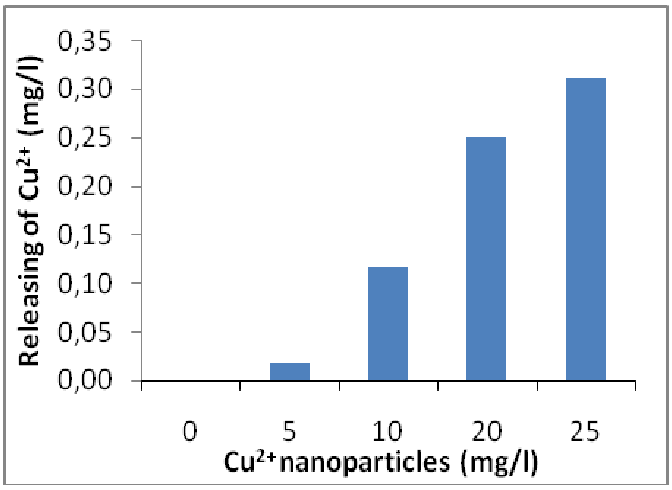

Figure 2. *Cu^{2+} release from N- CuO*

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Effects on growth rates

Anabaena growth in the presence of various concentrations of copper nanoparticles and salt is shown in *Figure 3*. The results indicate that cyanobacterial growth decreased by increasing the concentration of salt particles as compared with the control. The highest reduction occurred at 0.32 M NaCl and 25 mg/l CuO-NPs (*Fig. 3*). This was visually apparent, as seen by yellowing and fragmentation of filaments.

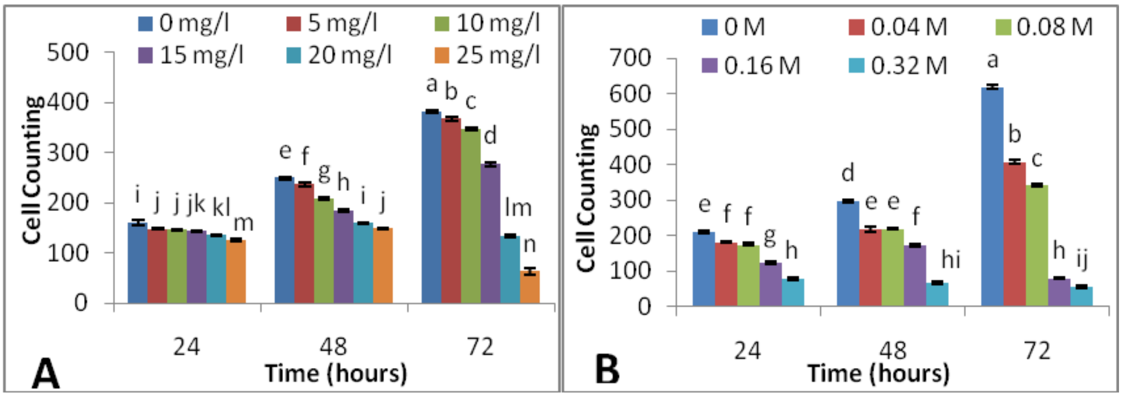

Figure 3. *Growth rate of Anabaena sp. after exposure to different concentrations of CuO-NPs (A) and NaCl (B).The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with p<0.05.*

The results of 72-hour toxicity tests, the 24, 48 and 72-hour levels of EC_{10}, EC_{30}, EC_{50} and EC_{90} for CuO-NPs and NaCl toxicity are summarized in *Tables 2* and *3*. The range of EC_{30} values varied from 177.82 mg L^{-1} (24 h) to 12.58 mg L^{-1} (72 h) for CuO-NPs tests and from 0.114 M (24 h) to 0.052 M (72 h) for NaCl.

Table 2. *The values of EC_{10}, EC_{30}, EC_{50}, EC_{90} and NOEC for CuO-NPs*

Time (hours)	EC_{10} (mg/l)	EC_{30} (mg/l)	EC_{50} (mg/l)	EC_{90} (mg/l)	NOEC (mg/l)
24	13.7	177.82	1047.12	79432.82	104.71
48	8.91	18.19	29.51	97.72	2.95
72	7.76	12.58	17.37	38.9	1.73

Table 3. *The values of EC_{10}, EC_{30}, EC_{50}, EC_{90} and NOEC for NaCl*

Time (hours)	EC_{10} (M)	EC_{30} (M)	EC_{50} (M)	EC_{90} (M)	NOEC (M)
24	0.035	0.114	0.248	1.07	0.024
48	0.03	0.085	0.168	0.928	0.016
72	0.022	0.052	0.095	0.398	0.095

The effects on non-enzymatic antioxidants

The content of protein and soluble sugars

Protein content in all CuO-treatments showed a significant decrease compared to the control (*Table 4*). The percentage decreases of protein, relative to the control value were

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13, 44.8 and 41% in NaCl, CuO and NaCl+CuO-treated cultures, respectively. However cellular contents of soluble sugars after treatment with NaCl and CuO-NPs+NaCl increased significantly (*Table 4*).

Table 4. *Content of protein, proline, soluble sugars, total phenol, flavonoids and MDA in Anabaena sp. under NaCl, CuO-NPs, NaCl+CuO-NPs treatments. The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with p<0.05.*

Column	Protein (mg/g FW)	Proline (mg/l)	Soluble Sugars (mg/l)	Total Phenol (mg/l)	Flavonoids (mg/l)	MDA (nmol/g FW)
Control	2.94± 0.25 a	6.27± 0.25 bc	15.65±0.45 b	13.5± 0.59 a	0.016± 0.0004 bc	6.25± 0.02 b
NaCl	2.6±0.34 ab	5.26± 0.02 c	63.47± 3.23 a	13.33± 1.24 a	0.015± 0.0002 c	9.89 ± 0.22 a
CuO	1.62± 0.39 b	10.21± 0.51 a	18.05± 0.1 b	13.6± 0.15 a	0.024± 0.0014 a	7.29± 0.29 b
NaCl+CuO	1.75± 0.12 b	7.42±0.44 b	65.96±1.83 a	13.87± 0.90 a	0.017± 0.0002 b	8.81 ± 0.63 a

The effect on malondialdehyde

Lipid peroxidation was assayed by measuring malondialdehyde (Zhanga et al., 2013). Changes of malondialdehyde in cyanobacteria *Anabaena sp.* under stress from copper nanoparticles, salinity and both stresses at the same time is shown in *Table 4*. Malondialdehyde content significantly increased under CuO-NPs and CuO-NPs+NaCl compared to control.

Proline content

Proline content in treatments with NaCl and NaCl+CuO-NPs showed no significant changes. However, in the CuO-NPs treatment, proline content increased 64.1 % at the 10.93 mg/l level of CuO-NPs (*Table 4*).

Total phenols and total flavonoids

Phenolic content did not differ significantly between the three types of treatment compared and with the control group. Flavonoid content significantly increased 63.1% in CuO treatment compared to the control, while there was no significant difference between control, NaCl and NaCl+CuO treatments.

The effect on enzymatic antioxidants

In this study, the enzyme activity of POD in treatment with CuO-NPs and NaCl was found to be significantly increased. In contrast, polyphenol oxidase (PPO), another enzyme that is involved in neutralizing ROS compounds and assayed in this study, was significantly reduced in all treatments compared to control. CAT activity showed a different behavior, with a significant increase compared to control (~600 %, $p < 0.05$) in treatment with NaCl+CuO as well as (~400 %, $p < 0.05$) in treatment with CuO, while it remained unchanged in NaCl treatments; and finally, the other peroxide scavenging enzyme, APX, was also found to be induced under CuO-NPs stress (*Table 5*).

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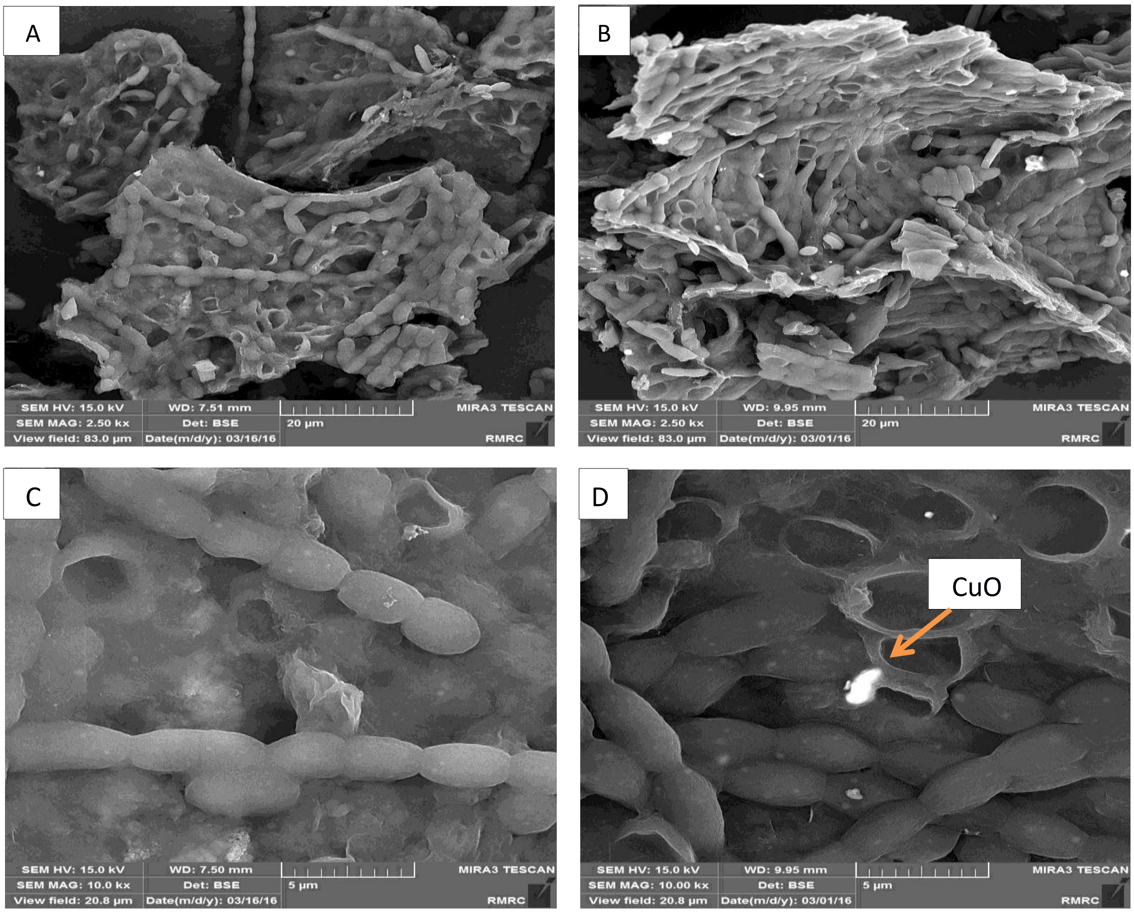
Table 5. *Enzymatic antioxidant content in Anabaena under NaCl, CuO-NPs, NaCl+CuO-NPs treatments. The data represents the average of three replicates ± standard error (SE). Different letters indicate significant differences among treatments according to Duncan's test with p<0.05.*

Factors / Treatments	POD (Unit/g FW)	PPO (Unit/g FW)	APX (Unit/g FW)	CAT (Unit/mg protein)
Control	0.008 ± 0.0006b	2200 ± 57.7a	0.446 ± 0.026c	0.0025 ± 0.0006c
NaCl	0.005 ± 0.0013a	1666.7 ± 33.3b	0.625 ± 0.026b	0.005 ± 0.0006c
CuO	0.009±0.0012a	1666.7 ± 33.3b	0.834 ± 0.015a	0.0095 ± 0.001b
NaCl+ CuO	0.016 ± 0.0013a	1000 ± 9c	0.521 ± 0.039c	0.0152 ± 0.0019a

Electron microscopy

CuO-NPs attached to the surface of the cyanobacterial cells in direct contact with them is demonstrated clearly by SEM images in *Figure 4*. Generally, electron microscopic images showed that the nanoparticles bound to each other and, more relevantly, to the surface of the cyanobacterial cells, altering the appearance of cells through disruption of direct physical interactions between the cells and increasing the number of lysed cells (*Fig. 4*, *A-E*).



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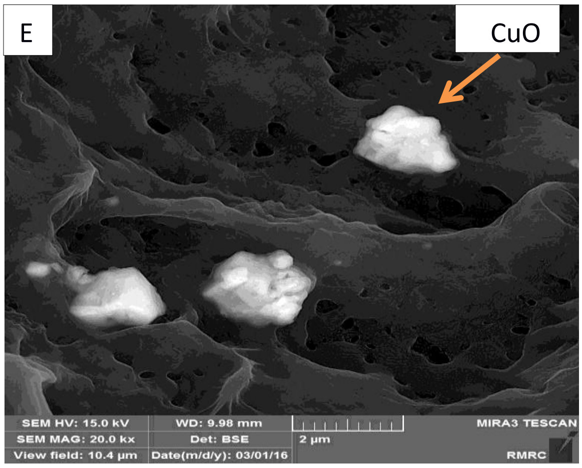

Figure 4. *SEM images of Anabaena sp. after 72 hours exposure to CuO-NPs at concentration of 10.93mg/l. Figures of A and C show control samples and figures B, D and E show Anabaena cells which treated with CuO-NPs.*

Discussion

As illustrated in *Figure 2*, small amounts of Cu^{2+}were released. This can be attributed to the aggregation of CuO-NPs and the slightly alkaline pH environment of the suspension (Wang et al., 2013). Cells cannot exclude Cu^{2+} and accumulate it in high quantity. Cu^{2+} becomes powerfully toxic for phytoplankton communities at concentrations between 0.1 and 1.0 mg/l (Deniz et al., 2011). The release of Cu^{2+} which is seen in our experiment is in this range too. Although algal cells are able to encase and detoxify Cu^{2+} but this can occur in restricted ranges (Toncheva et al., 2006). The decrease in the rate of cell division caused by metals has been primarily attributed to their binding to sulfhydryl groups which are important for regulating plant cell division (Visviki and Rachlin., 1991). Furthermore, The difference between effective concentrations ($EC_{10,30,50}$) in two treatments indicated that the degree of nanoparticle toxicity is also likely to depend on their nano structures and high surface to mass ratio as well as the nature of their constitutive element (Hernández Battez et al., 2010).This varies between algal species (Pendashte et al., 2013).The results of Shi (2011) have also indicated that CuO-NPs toxicity is three to four times higher than that of ionic Cu, because of the larger uptake of NPs-released Cu. Therefore, as it is shown in *Figure 2*, cell counting decreased during CuO-NPs treatment, compared to NaCl treatment.

As is shown in *Table 4*, percentages of proteins decreased in treated samples relative to control samples meaningfully. Rahman et al. (2011) observed similar findings in cyanobacteria under metal stress; Cu^{2+} treatment also resulted in reduction of protein content extensively. This decline may be due to production of ROS, which is known to damage protein, and therefore disturbs the cellular homeostasis. Similar observations were made, also, in *A. doliolum* exposed to cadmium and UV-B stressed (Bhargava et al., 2007). In contrast, adaptation to salt stress in cyanobacteria is generally accomplished by excretion of inorganic ions from the cells to balance osmotic potential; cells can also prevent denaturation of macromolecules via accumulation of some osmolytes, such as sucrose, trehalose, glucosylglycerol, glycine betaine, proline and/or glutamate (Allakhverdiev et al., 2005).

Lipid peroxidation is linked to the production of O_2^-. The presence of high amounts of transitional metals such as Cu (II) favors the enhanced generation of OH° from O_2^-

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through the Fenton reaction (Luna et al., 1994). Thus, the increased levels of MDA suggest that Cu (II) ions stimulated free radical formation in *Anabaena sp*.

Increases in proline concentration under heavy metal and salt stresses have been reported in some higher plants and in the cyanobacteria *Spirulina*, *Anabaena* and *Cylindrospermum* (Rahnama and Ebrahimzadeh., 2004; Chris et al., 2006; Choudhary et al., 2007). It seems that an adaptive mechanism via increase in proline is activated for neutralization of accumulated CuO-NPs and the acidity. Proline's chelating ability to bind metal ions can also be a defense mechanism for survival (Deniz et al., 2011).

It has been recently shown that accumulation of secondary metabolites, such as phenylpropanoids, including flavonoids, which has scarcely been reported in cyanobacteria, could be one of the strategies for cyanobacterial organisms to protect against cellular damage (Singh et al., 2014). Flavonoids, phenolic acids, and tannins, as well as some derivatives of flavonoids, are also compounds which have many different biological activities including antioxidant activity under stress conditions (Agati et al., 2011), in addition to some medicinal activities (Fresco et al., 2006). Although only scarce reports are available on total phenols and total flavonoid content under salt and nano particle stress tolerance responses in cyanobacteria, positive correlations with the accumulation of polyphenolics, enhanced antioxidant activity and tolerance to stresses have been reported in plant species (Korkina, 2007). As a result, there is no additive response of non-enzymatic antioxidant factors in NaCl+CuO-NPs combination treatment compared to their responses to single stresses. This may be due to their antagonistic effects (Rai et al., 2013).

During oxidative stress, POD is a very important enzyme able to remove the dangerous radical products of superoxide and hydrogen peroxide (Beier et al., 1991). The greater activity of POD in treated compared to control plants can be attributed to stress caused by the treatments (Cosio and Dunand, 2010). These results suggest that NaCl and CuO-NPs individually as well as NaCl+CuO-NPs can be very important stressors impacting plant physiology. Reduction of PPO in both NaCl and CuO-NPs-treated cells was more than other treatments. In our study, CAT activity significantly increased compared to control in all treatments of CuO-NPs. Free Cu^{2+} can catalyze the formation of highly toxic ROS such as hydroxyl radicals ($OH^{\bullet}$) from superoxide anions ($O2^{\bullet-}$) or hydrogen peroxide (H_2O_2) (Melegaria et al., 2013). Therefore, increase in CAT activity of treated samples compared to control very probably is a response to substantial ROS production. APX induction may be attributed to the *apx* operon induction by H_2O_2 production (Vranova et al., 2002). Therefore, it seems that H_2O_2 can be detoxified by a combined effort of POD, CAT and APX in *Anabaena sp*. That enhanced levels of most antioxidant enzymes, CAT, POD and APX, were observed for *Anabaena sp.* in this study indicates the protective role of these enzymes against copper-induced oxidative stress (*Table 5*). Finally, it can be noted that, except for CAT, which shows more activity in NaCl+CuO-NPs treatments compared to NaCl and CuO-NPs treatments, there were no additive activities in other enzymatic antioxidants.

CuO-NPs often increased cell aggregation by forming bridges between the cells in the areas where the nanoparticles had accumulated. The Cuo-NPs attached to the surface of the cyanobacterial cells and the direct contact is demonstrated clearly by the SEM images in *Figure 4*. Their external surfaces had begun to fracture compared with the surfaces of cells that were not in contact with these particles. The structure of the cell wall was probably affected by the metal ions (*Fig. 4E*). Similarly, others have found cellular ultrastructural changes in *Microcystis aeruginosa* which were exposed to Ag-

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NPs (1 mg l^{-1}) (Duong et al., 2016). After 48 h exposure with Ag-NPs the structure of *Microcystis* was changed; the cells were shrunken and distorted. Other studies on the impact of nanoparticles on other species have yielded similar results (Ayatallahzadeh Shirazi et al., 2015).

Conclusion

The outcomes of this study suggest that single stresses of NaCl and CuO-NPs can have inhibitory effects on *Anabeana sp.* in the used ranges, inducing severe damages in high concentrations. However, stress induced by these factors in combination did not result in elevated inhibitory effects, more than those observed in single stresses. This may be related to interactions between released ions from NaCl and CuO-NPs.

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AGRONOMIC BIOFORTIFICATION OF GREEN BEAN (*PHASELOUS VULGARIS* L.) WITH ELEMENTAL SULPHUR AND FARMYARD MANURE

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Abstract. The effects of elemental sulphur and farmyard manure on agronomic biofortification within the parameters of N, P, S and N:S ratio were evaluated in green bean (*Phaselous vulgaris* L.). Sulphur 0 (S_0), 50 (S_1), 100 (S_2), 150 (S_3), 200 (S_4), 400 (S_5) mg kg^{-1} and farmyard manure 0 (FYM_0), 3 (FYM_1) tonnes da^{-1} were applied to a calcareous sandy loam soil with low organic matter and sulphur deficient. Three weeks after the applications, bean seeds were sown in pot soils and after eight weeks of growing period the shoots were harvested. The soil pH was decreased while EC was increased by the applications of S and FYM. The P concentration of shoot was increased by S with FYM. While the dry weight and S concentration of shoot were increased, N concentration was slightly decreased by S, alone. The N:S ratio decreased from 23.76 in S_0FYM_0 to 15.93 in S_5FYM_1. All results indicate that sulphur applications in S_1 and S_2 levels with farmyard manure can be sufficient for growing bean in the soil.
Keywords: *malnutrition, chemical fertilizer, organic manure, nitrogen, phosphorus*

Introduction

Two thirds of the world population faces with one or more essential mineral deficiencies in their nutrition. Solutions to this problem are being investigated by means of dietary diversification, mineral adding, food supplements or increasing bioavailability and concentrations of mineral elements during crop production. Especially there have been recent studies on plants which have an essential importance in human nutrition with agronomic and genetic biofortification. Agronomic biofortification tries to ensure the optimization of mineral fertilizer applications and improving of mineral element mobility and solubility in soil. The optimization of chemical fertilizer and organic manure applications or the applications of essential elements with enriched fertilizer (especially NPK fertilizers) throughout soil, leaf or seed during cultivation should not only provide a quick solution in human and animal nutrition but also will be a complementary approach for ongoing plant breeding programme.

Sulphur is one of the essential elements in plant growth. Plants need sulphur for protein synthesis, chlorophyll, oil and vitamin formation. Sulphur deficiency has a negative effect on both yield and quality of crops which are grown for human and animal consumption (Tiwari et al., 1997). The specific aim of most biofortification studies is to reduce mineral, vitamin and protein deficiencies in edible parts of plants. Sulphur takes role in synthesis of some compounds such as cysteine, cystine and methionine for protein formation in plants. Low sulphur content in proteins is a nutritionally adverse situation and the methionine which is the main source of protein

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and can be synthesized in the presence of sulphur is a basic amino acid for human nutrition.

Sulphate absorbed by the roots is the most important source of sulphur. Despite the sulphur's distribution on all parts of soil profile, it has at its highest concentration on surface soil which is rich in organic matter. Sandy or low organic content soils are the ones which are encountered with sulphur deficiency. On the situations of high efficiency on crop yield, especially on sandy soils and soils that are poor in organic matter which has abundant watering, the sulphur content is not in sufficient quantity for the needs of plants (Anonymous, 1982). The sulphur deficiency in plants can also occur on coarse textured soils having a high pH and when there is insufficient moisture on their root zone (Tiwari, 1995). And also the sulphur deficiency usually occurs on cultivation areas where chemical fertilizers are used and of the plants that are in highly need of sulphur due to their high yield capacity. Therefore, in addition to nutrients such as N, P_2O_5 and K_2O, S which is also named as the fourth basic macro nutrient should also be considered while doing fertilization programmes.

Bean is not only a product of high nutritional value, but also is an important vegetable protein source for humans. It has been widely consumed in Turkey and in Asian, African, European, North, South and Central American countries. For this reason, it is essential that it takes an important role among plants which are to be studied for increase yield and nutritive value. In this study, the effect of sulphur and farmyard manure applications on calcareous sandy loam soil having a low-sulphur and organic matter content for agronomic biofortification in bean is being examined using parameters of N, P, S and N:S.

Material and methods

Experimental setup

Soil was air dried and passed through a 4 mm sieve. A total of 9 kg of sieved soil was placed in pots with holes at the bottom. Sulphur (S_0:0, S_1:50, S_2:100, S_3:150, S_4:200, S_5:400 mg kg^{-1}) without farmyard manure (FYM_0: 0 tonnes da^{-1}) and with farmyard manure (FYM_1: 3 tonnes da^{-1}) was applied to soil according to completely randomized design factorial with 4 replicates. At the time of the application materials were mixed to soil, soil samples were taken at 1st sampling period. Then, basal fertilization (as 8 kg N da^{-1}, 8 kg P_2O_5 da^{-1} and 8 kg K_2O da^{-1}) using 15.15.15 fertilizer was made to each pot soil. Each pot was equally watered and incubated for 3 weeks. The green bean 'Öz Ayşe' was used as an experimental plant. At the end of the 3 weeks, 2nd period soil samples were taken and four green bean seeds were sown in each pot soil. After the seeds germination, one bean plant was left in per pot and the plants were grown for 8 weeks. During the vegetation period, N 8.5 kg da^{-1} (NH_4NO_3, 33% N), P_2O_5 3.7 kg da^{-1} (MAP), K_2O 10 kg da^{-1} (KNO_3), MgO 0.62 kg da^{-1} ($MgNO_3$) and 1.73 kg microelement fertilizer da^{-1} (Hortrilon, 5% Fe, 2.5% Mn, 0.5% Zn, 2.5% Cu) were applied to each pot as fertilizer solution. At the end of the experiment, the shoots were harvested and 3rd period soil samples were taken from each pot.

Plant analysis

The shoots were washed with deionized water and then dried 65 $^{\circ}$C for 72 h to determine dry weight. The dried shoots were ground in a stainless steel mill which

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enabled them to be passed through a 20 mesh screen. The samples of 0.5 g each were digested with 10 mL HNO_3 and $HClO_4$ (4:1) acid mixture on a hot plate and filtered and then, diluted to 100 mL using distilled water. Total S was conducted by the turbidimetric method with $BaCl_2{\cdot}2H_2O$ and the readings were taken using a spectrophotometer at 430 nm (Kacar and Inal, 2008). Total P was measured by a modified colorimetric molybdo-vanado-phosphate method using a spectrophotometer at 430 nm (Kacar and Inal, 2008). Total N was determined by a modified Kjeldahl procedure (Kacar and Inal, 2008).

Soil analysis

The soil samples were air-dried and passed through a 2 mm sieve. The pH was measured in H_2O (1:2.5 soil:deionized water) and the electrical conductivity (EC) value was determined directly on the saturation paste. The soil particle size analysis was done by using the hydrometer method (Bouyoucos, 1955) and the $CaCO_3$ content was determined by using a Scheibler calcimeter. Organic matter was determined by using modified Walkley-Black procedure (Black, 1965). The total nitrogen was done by using modified Kjeldahl procedure (Kacar, 2009). Available P was extracted by $NaHCO_3$ and determined by a molybdate colorimetric method (Olsen and Sommers, 1982). Soil samples were extracted for SO_4-S by using 500 mg kg^{-1} P as KH_2PO_4 which contents of Fox et al. (1964). Analyses were conducted by the turbidimetric method with $BaCl_2{\cdot}2H_2O$ and the readings were taken using a spectrophotometer at 430 nm (Kacar, 2009). Some of the physical and chemical properties of experiment soil were given in *Table 1*.

Table 1. *Physical and chemical properties of soil used*

Parameter	Soil
Sand (%)	63.52
Clay (%)	18.48
Silt (%)	18
Texture	Sandy loam
pH	7.80
$CaCO_3$ (%)	18.92
EC (dS m^{-1})	2.07
Organic matter (%)	2.27
Total N (%)	0.049
Available-P (mg kg^{-1})	34.57
Extractable-SO_4-S (mg kg^{-1})	2.40

Statistical analysis

The data were analyzed by standard ANOVA procedures and their significances were always based on the $P<0.05$ level using the LSD tests.

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Results and discussion

Effects of sulphur and farmyard manure on soil pH and EC

In the 1st soil sampling period, the farmyard manure caused an important but little increase of 0.03 units on soil pH. In the 2nd and 3rd soil sampling periods, the effects of interactions of sulphur and farmyard manure on soil pH were found significant. In the 2nd sampling period, the highest decrease on soil pH was respectively obtained as 7.65 and 7.64 in applications of S_5FYM_0 ve S_5FYM_1. When the sulphur applications whether with or without farmyard manure compared, the applications of S_0FYM_0 and S_0FYM_1, S_2FYM_0 and S_2FYM_1, S_4FYM_0 and S_4FYM_1 differed from each other and in these applications, soil pH was determined to be a little bit lower when sulphur is applied with farmyard manure. And in the 3rd soil sampling periods, the highest decrease on soil pH was respectively obtained as 7.70 and 7.76 in applications of S_5FYM_0 and S_5FYM_1. When the sulphur applications with or without farmyard manure compared, S_2FYM_0 and S_2FYM_1, S_5FYM_0 and S_5FYM_1 differed from each other and in these applications, a little increase in soil pH was obtained when sulphur is applied with farmyard manure. In an overall evaluation, especially in the 2nd sampling period (after three weeks from applications) depending on irrigation, the pH of control soil (S_0FYM_0) rose from 7.81 up to 8.01, whereas no increase in sulphur with farmyard manure applications was observed. Almost similar effects of the applications were determined in the 3rd sampling period (*Table 2*). Kaplan and Orman (1998) reported that the sulphur applied to high calcareous soil decreased pH in units of 0.07-0.35 and the pH started to rise again depending on time.

Table 2. *Effects of sulphur (S) and farmyard manure (FYM) on soil pH*

Sulphur doses	Soil pH								
	1st soil sampling			**2nd soil sampling**			**3rd soil sampling**		
	FYM_0	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**
S_0	7.81	7.83	7.82	8.01 a(1), **A**	7.88 a, **B**	7.95	7.89 a, **A**	7.90 a, **A**	7.89
S_1	7.85	7.86	7.85	7.83 b, **A**	7.85 a, **A**	7.84	7.81 b, **A**	7.83 b, **A**	7.82
S_2	7.83	7.87	7.85	7.83 b, **A**	7.74 b, **B**	7.79	7.77 c, **B**	7.80 c, **A**	7.78
S_3	7.81	7.87	7.84	7.74 c, **A**	7.75 b, **A**	7.75	7.78 c, **A**	7.79 c, **A**	7.78
S_4	7.83	7.85	7.84	7.76 c, **A**	7.72 b, **B**	7.74	7.76 c, **A**	7.75 d, **A**	7.76
S_5	7.83	7.87	7.85	7.65 d, **A**	7.64 c, **A**	7.64	7.70 d, **B**	7.76 d, **A**	7.73
Means	7.83 **B**(2)	7.86 **A**		7.80	7.76		7.78	7.80	
ANOVA(3)									
S	n.s.			**			**		
FYM	**			**			**		
S*FYM	n.s.			**			*		

(1)Means in the same column followed by the same letter are not significantly different at 5% probability level by LSD test; (2)Means in the same row followed by the capital and bold same letter are not significantly different at 5% probability level by LSD test; (3)Significance levels: *p<0.05; **p<0.01; n.s.: non significant

In the 1st soil sampling period, the effects of sulphur on soil EC was not significant whereas the farmyard manure effect was important. While the soil EC was highly affected from the sulphur and farmyard manure in the 2nd and 3rd sampling periods, the

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interaction between these two applications was not significant in each three sampling periods. The farmyard manure caused an increase about 0.29, 0.37 and 0.29 units in soil EC, respectively in the 1st, 2nd and 3rd sampling periods. The sulphur yet caused an increase on soil EC only in the 2nd and 3rd sampling periods and the highest increase of EC was observed in S_4 and S_5 (*Table 3*). The sulphur applications are reported to cause an increase in soil salinity in several studies (Modaish et al., 1989; Kaplan and Orman, 1998; Orman and Kaplan, 2011; Orman and Ok, 2012). And also in this study, it has been indicated that especially the sulphur applications increases the soil salinity significantly. It is thought that this is because of the SO_4^{-2} having been formed in soil after the sulphur applications and also because of irrigation and fertilization during the growing period. Thus, a significantly positive correlation between EC values and SO_4^{-2}-S concentrations of greenhouse soils where tomato cultivation is made in West Mediterranean Region has been reported by Orman and Kaplan (2009).

Table 3. *Effects of sulphur (S) and farmyard manure (FYM) on soil EC*

Sulphur doses	Soil EC (dS m^{-1})								
	1st soil sampling			**2nd soil sampling**			**3rd soil sampling**		
	FYM_0	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**
S_0	2.10	2.33	2.22	4.35	5.01	4.68 c(1)	3.45	3.61	3.53 d
S_1	2.26	2.55	2.41	5.20	5.85	5.52 b	4.05	4.05	4.05 c
S_2	2.25	2.50	2.38	5.26	5.61	5.44 b	4.26	4.32	4.29 bc
S_3	2.13	2.49	2.31	5.34	5.55	5.44 b	4.07	4.26	4.16 c
S_4	2.20	2.55	2.37	5.77	6.23	6.00 a	4.07	5.00	4.53 ab
S_5	2.17	2.49	2.33	6.43	6.34	6.38 a	4.64	5.02	4.83 a
Means	2.22 **B**(2)	2.48 **A**		5.39 **B**	5.76 **A**		4.09 **B**	4.38 **A**	
ANOVA(3)									
S	n.s.			**			**		
FYM	**			**			**		
S*FYM	n.s.			n.s.			n.s.		

(1)Means in the same column followed by the same letter are not significantly different at 5% probability level by LSD test; (2)Means in the same row followed by the capital and bold same letter are not significantly different at 5% probability level by LSD test; (3)Significance levels: **p<0.01; n.s.: non significant

Effects of sulphur and farmyard manure on bean plant

The effects of applications on total nitrogen, sulphur, and phosphorus concentrations, N:S ratio and dry weight in the shoots of bean were given in *Table 4*.

The sulphur alone is found important to have a significant effect on nitrogen concentration in shoots an especially in higher doses of 100 mg S kg^{-1}, there has been a reduction in nitrogen concentration. Schung (1990) specified that the increase of S content in plant tissues with suitable sulphur fertilization in vegetables had been effective in decrease of NO_3 content in tissues. It was reported that sulphur applied to soil has no significant effect on nitrogen concentration in tomato plant, but the N concentration is observed to be lower than the control (Orman and Kaplan, 2011). Gaines and Phatak (1982) reported that with the increase of the sulphur ratio applied to soybean grown in hydroponic culture, the N concentration decreased in a significant

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rate. In our study, the decrease in nitrogen concentrations of bean shoots by the sulphur applications can either be related with sulphur and nitrogen relationships, or just arise due to the dilution effect of nitrogen in result of dry weight increase by sulphur.

Table 4. *Effects of sulphur (S) and farmyard manure (FYM) on shoot N, S, P concentrations, N:S ratio and dry weight*

Sulphur doses	**N (%)**			**S (%)**			**P (%)**			**N:S**			**Dry weight (g pot^{-1})**		
	FYM_0	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**	**FYM_0**	**FYM_1**	**Means**
S_0	3.24	3.22	3.23ab[1]	0.14	0.17	0.16c	0.28c,**B**[2]	0.32a,**A**	0.30	23.76a,**A**	18.72bc,**B**	21.24	21.56	23.01	22.28b
S_1	3.30	3.25	3.27ab	0.17	0.17	0.17bc	0.34a,**A**	0.33a,**A**	0.33	19.87b,**A**	19.82b,**A**	19.84	28.61	28.54	28.57a
S_2	3.42	3.33	3.37a	0.19	0.19	0.19a	0.31b,**B**	0.34a,**A**	0.33	18.83bc,**A**	17.25bcd,**A**	18.04	28.76	24.72	26.74a
S_3	3.00	3.22	3.11b	0.18	0.17	0.18ab	0.31b,**A**	0.32a,**A**	0.32	16.53cd,**A**	19.03bc,**A**	17.78	27.17	28.99	28.08a
S_4	3.12	3.27	3.20ab	0.19	0.19	0.19a	0.33ab,**A**	0.33a,**A**	0.33	16.63cd,**A**	17.22bcd,**A**	16.93	26.13	27.44	26.78a
S_5	3.15	3.09	3.12b	0.19	0.20	0.19a	0.35a,**A**	0.33a,**A**	0.34	16.85cd,**A**	15.93d,**A**	16.39	27.36	27.96	27.66a
Means	3.20	3.23		0.17	0.18		0.32	0.33		18.74	17.99		26.60	26.78	
ANOVA[3]															
S	*			**			**			***			**		
FYM	n.s.			n.s.			*			n.s.			n.s.		
S*FYM	n.s.			n.s.			**			**			n.s.		

[1]Means in the same column followed by the same letter are not significantly different at 5% probability level by LSD test; [2]Means in the same row followed by the capital and bold same letter are not significantly different at 5% probability level by LSD test; [3]Significance levels: ** p<0.01; n.s.: non significant

The sulphur, farmyard manure and sulphur with farmyard manure interaction had a great impact on shoot phosphorus concentration. The shoot phosphorus concentration is determined as 0.28% in S_0FYM_0 whereas it is seen as 0.35% in S_5FYM_0. Having been evaluated with or without farmyard manure, the applications of S_0FYM_0 and S_0FYM_1, S_2FYM_0 and S_2FYM_1 differed from each other statistically and in these applications, applying sulphur with farmyard manure caused an increase in shoot phosphorus concentration. Also, it is thought that shoot phosphorus concentration increases due to the decrease in soil pH because of consequent sulphur applications. Brahim et al. (2017) indicated that the combined application of rock phosphate and elemental sulphur was the potential to improve the P nutrition of soybean.

The applications of sulphur alone significantly affected on shoot sulphur concentration. There has been an increase between 5.9% and 15.79% in shoot sulphur concentration according to the S_0. This impact is especially determined in 100 mg S kg^{-1} level. Even though the shoot sulphur concentration has an increase about 5.56% by farmyard manure application, this impact is not found important statistically. Tiecher et al. (2012) observed that the amount of S accumulated in the shoots of sunflower, bean, soybean and castor bean and the level of available S increased due to S fertilization.

Reuther and Robinson (1998) notified that bean plant in early stage has a sufficient S content of 0.16%-0.64% in shoots. The sulphur concentrations of shoots in our study range from 0.14% to 0.20%. According to the limit values, it is significantly output that

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there has been an improvement in sulphur nutrition level of bean, especially as a result of sulphur applications.

The sulphur alone and sulphur with farmyard manure applications were significantly affected on total N: total S ratio of shoot. The N:S ratio in shoot is assigned as 23.76 in S_0FYM_0 and 18.72 in S_0FYM_1 and it showed statistically significant differences. The other applications of sulphur with or without farmyard manure were statistically included in the same group. The highest ratio of N:S was determined in S_0FYM_0 application and the lowest of that was in S_5FYM_1 application. The shoot N:S ratio showed a significant reduction especially as a result of sulphur alone applications. Barczak and Nowak (2015) reported that the potato plants fertilised with sulphur, in comparison with the control, N:S ratio was narrowed due to a higher increase in the content of sulphur rather than nitrogen in potato tubers.

Although it has been obtained that the effects of sulphur with farmyard manure has a great role on the shoots N:S ratio, it is thought that the determining factor in the decline of this ratio is the sulphur applications. Total N: Total S ratio of the leguminous for protein synthesis is reported to be 17 (Kacar and Katkat 2007). It is pointed out by Stewart and Porter (1969) that the sulphur deficiency may be limiting in protein formation in case of the total N:total S ratio's being over 16 and it is just a severe sulphur deficiency in case of the ratio's being over 20 or more. The soil SO_4^{-2}-S concentration below 10 mg kg^{-1} is considered to be deficient (Radish et al., 1995). According to this limit value, the extractable SO_4^{-2}-S (2.40 mg kg^{-1}) concentration of our experiment soil appears to be deficient. In this case, N:S ratio designated as 23.76 in S_0FYM_0 application is the indicator of the plant's facing with a deficiency in its sulphur nutrition and this will obviously cause negative effects in protein synthesis. However, by the increase of the sulphur applications, the imbalance of plant's N:S ratios disappeared and it seems to have a balance between nitrogen and sulphur elements which are highly important in protein synthesis. The uptake of sulphur, yield, quality and optimizing the N:S ratio in plants were improved by sulphur fertilization (Skwierawska et al., 2016). But also while trying to maintain this balance, the risk of sulphur's increasing soil salinity should be kept in mind and it should be taken into consideration while doing sulphur fertilization especially on saline soils. A greenhouse survey study in Antalya/Turkey, it appears that there are imbalance for N:S ratio, even if a sulphur deficiency is not determined in tomato leaves (Orman and Kaplan, 2009)

The effect of sulphur alone is found to be important on shoot dry weight. The dry weight was 22.28 g pot^{-1} in S_0 application whereas there became an increase about 17-22% by sulphur applications. The increase occurred was not due to the increase of sulphur level, the sulphur application doses statistically took place in the same group. Crusicol et al. (2006) indicated that sulphur fertilization has not considered by bean (*Phaseolus vulgaris* L.) growers. Whereas, the increase of grain yield can be limited by low levels of sulphur fertilization in common bean crops with high-input technology. Researchers reported that leaf S content, dry weight and grain yield of common bean is increased by sulphur fertilization. Kaplan and Orman (1998) reported an increase in dry weight of sorghum with sulphur applications.

Conclusion

The sulphur is an advisable material in order to decrease the soil pH on calcareous sandy loam and high pH soils and also to increase the uptake of the phosphorus element

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by plants of which the solubility in soil is significantly depending on pH. But the risk of the sulphur's creating salinity in soil should be taken into consideration and this negative effect should be well managed especially on the agricultural lands under the risk of salinity. Also, Gulmezoglu et al. (2016) reported that salt application generally reduced the fresh and dry weights of green bean genotypes.

On the sulphur deficient soils, the sulphur or other sulphur fertilizers absolutely should be included on fertilization programmes to obtain sulphur for the plants that are in need of sulphur requirement such as bean. Because it has been determined that if the necessary sulphur applications are not done on such soils, a significant imbalance may emerge in N:S ratio of the plant and the balance may be damaged against sulphur. When considering not only nitrogen but also sulphur as well is effective in protein formation in plants, this situation might have negative repercussions on high protein content plant's protein synthesis and quality such as bean.

According to the data obtained from this study, the applications of sulphur in 50 mg kg^{-1} and 100 mg kg^{-1} levels to bean growing on a calcareous sandy loam soils which are deficient in organic matter and SO_4^{-2} are thought to be sufficient in terms of yield and quality. Consequently, the further studies are needed to optimize amount of sulphur addition for other soil types and plant species.

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POTENTIAL NUTRITIVE VALUE OF *ASTRAGALUS* SPECIES HARVESTED AT THREE DIFFERENT MATURITY STAGES

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Abstract. The present study was conducted to investigate the effects of maturity stages on the chemical composition, in vitro gas and methane production, metabolic energy and organic matter digestibility for eleven *Astragalus* species widely encountered over the rangelands and intensely grazed by ruminants. *Astragalus* samples were collected from the rangelands at three different stages namely as before flowering, flowering and bear fruit stages. Dried samples were then subjected to various chemical analysis. Effects of *Astragalus* species and maturity stages on chemical composition, in vitro gas and methane production, metabolic energy and organic matter digestibility were found to be quite significant ($P < 0.001$). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) ratios increased, while the crude protein, crude ash, crude oil, condensed tannin contents, gas and methane production levels decreased with the progress of maturity stage. It can be suggested that grazing was more favorable at before flowering or flowering stages of *Astragalus* species because of high crude protein and metabolic energy content of plant at these stages. Also, although all *Astragalus* species were considered as a quality feed source for ruminants, endemic *Astragalus oocephalus* and *Astragalus longifolius* species were prominent with their superior nutritive properties.
Keywords. *chemical composition; in vitro gas production; metabolic energy; digestibility; condensed tannin content; grazing*

Introduction

Astragalus with about 2000 species is a member of legumes family and commonly composed of herbaceous and small shrubs. They are used in animal feeds, medicines and pharmaceuticals, in erosion prevention, bee pastures, dye and textile industries (Gruenwald et al., 1998). *Astragalus* species are widespread in steppes with low precipitation levels and Alpines with low temperatures and commonly grazed by

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ruminants. There is limited information about the nutritional properties and grazing periods of *Astragalus* species.

Harvest or grazing periods greatly influence nutritive values of feeds. Studies about harvest or grazing periods of these species are of great significant for yield and quality of feed and also for sustainable use of natural resources. Despite these kinds of investigations on several species, there are still a lot of different plant species to be researched (Kamalak and Canbolat, 2010; Kaplan et al., 2014a).

Chemical composition, metabolic energy and digestible nutrients are the significant quality indicators for animal feeds (Canbolat, 2012). For this purpose, in vitro gas production technique developed by Menke et al. (1979) is widely used in recent years to determine nutritional values of feeds under in vitro conditions since the technique is a fast, easy and cheap method (Kaplan et al., 2014b). The gas production technique is also used to determine the methane reduction potential of feeds affecting global warming (Lin et al., 2013). The present study was conducted to investigate the effects of maturity stages on chemical composition, in vitro gas and methane production of different *Astragalus* species.

Materials and methods

Plant materials

Eleven different *Astragalus* species widespread in Bingol province of Turkey and commonly grazed by ruminants were harvested from the rangelands and natural areas at three different maturity stages (before-flowering, full-flowering, fruit-set). *Astragalus gummifer* Labill, *Astragalus cinereus* Willd, *Astragalus compactus* Lam, *Astragalus lineatus* Lam. var. *longidens, Astragalus oocephalus* Boiss subsp. *stachyophorus, Astragalus amblolepis, Astragalus declinatus* Wild, *Astragalus lineatus* Lam. var. *lineatus, Astragalus longifolius* Lam., *Astragalus aureus* Willd and *Astragalus onobrychis* species were used as the plant materials of the study. From these species *Astragalus oocephalus* Boiss subsp. *stachyophorus* and *Astragalus longifolius* Lam. are endemic species.

Climate data

Climate data for Bingol province were received from the nearest meteorological station of Directorate of Meteorology. According the meteorological data of research area, the long-term (2000-2015) monthly average temperature is 12.3 °C, total precipitation is 917.8 mm and relative humidity is 56.6%. Average temperature, precipitation and relative humidity of the research period (August 2015-August 2016) were quite close to long-term averages (13.9 °C, 923.7 mm and 53.4%, respectively). Soil structure of the Bingol province is clay-loam and loamy (Ates and Turan, 2015).

Chemical analysis

The *Astragalus* species were harvested at before flowering, flowering and bear fruit stages. Samples were dried at 70 °C for 48 h, ground and sieved through 1 mm sieve. Crude ash content of samples was determined by ashing the samples at 550 °C for 8 h in an ash oven. Soxhlet extracting system was used to determine the crude oil content of the samples by ether extraction method (AOAC, 1990). Kjeldahl method was used to determine nitrogen (N) content of the *Astragalus* species and crude protein level was

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calculated by multiplying N content by 6.25 constant (AOAC, 1990). NDF (Van Soest and Wine, 1967) and ADF (Van Soest, 1963) analysis were performed by using ANKOM 200 Fiber Analyzer (ANKOM Technology Corp. Fairport, NY, USA). Condensed tannin content analysis of the samples was performed by using Butanol-HCl technique (Makkar et al., 1995).

The in vitro total gas and methane production

In vitro gas production technique developed by Menke et al. (1979) was used to determine in vitro gas and methane production of the samples. The rumen liquid was collected from three fistula installed hogget fed with a mixture 60% alfalfa and 40% barley. Liking blocks and clean water was supplied ad-libutum to the hogget. The rumen liquid was collected before feeding in the morning, filtrated through 6-layer cheesecloth to remove solid particles and mixed with twice as much synthetic saliva solution. Approximately 200 mg ground samples were placed in 100 ml glass syringes. The samples were weighed in triplicates. The sample syringes were then supplemented with 30 ml buffered rumen liquid. Three syringes including only buffered rumen liquid as control group and the syringes including samples and buffered rumen liquid were placed into a water bath set at 39 °C. The net gas production was calculated by subtracting gas production of control group from the gas production of sample group. Total gas production was determined as mL after incubation of *Astragalus* samples at 39 °C for 24 h. The methane ratio of the gas was obtained by using an Infrared methane analyzer (Sensor Europe GmbH, Erkrath, Germany) and the following equation (*Eq. 1*) was used to calculate methane production levels (Goel et al., 2008):

$$\text{Methane production}\ (\text{mL}) = \text{Total gas}\ (\text{mL}) \times \text{Methane ratio}\ (\%) \quad \text{(Eq. 1)}$$

Metabolic energy (ME) and organic matter digestibility (OMD)

Metabolic energy content of *Astragalus* samples was calculated in accordance with the following equation (*Eq. 2*) using some parameters related to 24 h gas production and chemical composition of samples (Menke and Steingass, 1988). Organic matter digestibility (%) of the samples was determined by using *Eq. 3* as suggested by Menke et al. (1979):

$$\text{ME}\ (\text{MJ kg}^{-1}\text{DM}) = 2.20 + 0.136\ \text{GP} + 0.057\ \text{CP} + 0.002859\ \text{CP}^2 \quad \text{(Eq. 2)}$$

$$\text{OMD}\ (\%) = 14.88 + 0.889\ \text{GP} + 0.45\ \text{CP} + 0.0651\ \text{CA} \quad \text{(Eq. 3)}$$

In these equations; DM: Dry matter, GP: Net gas production after 24 h (mL), CP: Crude protein ratio (%), CO: Crude oil ratio (%), CA: Crude ash ratio (%) and OMD: Organic matter digestibility (%).

Statistical analysis

The experimental design was completely randomized design with 3 replications. Variance and correlation analysis were performed by using Jump SAS (2009) statistical software. Tukey test was used to determine the difference between the means. As a complement of ANOVA procedure, Biplot Analysis was performed using nutritive

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value and gas production parameters as variables and *Astragalus* species as classification criterion (Yan and Kang, 2003).

Results

Chemical composition

The effects of harvest time on nutritional characteristics of different *Astragalus* species were investigated in this study. Chemical composition of *Astragalus* samples is provided in *Table 1*. The effects of *Astragalus* species, harvest time and *Astragalus* x harvest time interaction on chemical composition were found to be highly significant ($P \leq 0.01$). ADF and NDF ratios increased and crude protein, crude ash, crude oil and condensed tannin contents decreased with the progress of harvest time. Crude protein ratios varied between 11.65-32.79%, ADF ratios between 16.22-48.54%, NDF ratios between 35.62-66.08%, crude oil contents between 0.40-3.43%, crude ash contents between 3.14-11.54% and condensed tannin contents between 0.32-1.00%.

Table 1. *Chemical composition of Astragalus species harvested at different stages*

Astragalus species	Stages	CP	ADF	NDF	CT	CO	CA
Astragalus gummifer Labill.	Before flowering	21.7 e	26.05m	41.14mn	0.62g-j	1.98cd	6.39i-l
	Flowering	19.42 gh	28.14l	42.39lm	0.52l-o	1.31g-k	5.62lmn
	Bear fruit	14.05 r	32.45j	45.01k	0.40pq	0.92lmn	5.36m-p
	Average	**18.39 I**	**28.88 H**	**42.85 G**	**0.51 G**	**1.40 D**	**5.79 D**
Astragalus cinereus Willd.	Before flowering	24.82c	23.18o	36.43p	0.87b	2.36b	8.58cde
	Flowering	22.41de	28.63kl	39.32o	0.66fg	1.5fgh	7.65efg
	Bear fruit	19.9fg	33.69i	41.80m	0.49mno	0.87mno	6.93g-k
	Average	**22.37 C**	**28.50 I**	**39.19 I**	**0.68 C**	**4.58 C**	**7.72 B**
Astragalus compactus Lam	Before flowering	21.91e	42.43d	52.93f	0.55j-m	1.13kl	5.14n-q
	Flowering	18.23i-l	46.19c	57.3e	0.46op	0.82nop	4.58o-r
	Bear fruit	17.26lmn	47.29b	63.04b	0.39q	0.59pq	3.60 rst
	Average	**19.13 H**	**45.30 A**	**57.76 B**	**0.47 H**	**0.85 G**	**4.44 F**
Astragalus lineatus Lam. var. *longidens*	Before flowering	22.11e	33.23ij	42.17m	0.98a	1.71ef	7.96def
	Flowering	19.32ghi	37.23g	47.95j	0.76cde	1.14jkl	7.23f-i
	Bear fruit	17.99jkl	39.97e	51.28gh	0.54k-n	0.93lmn	6.4i-l
	Average	**19.81 F**	**36.81 D**	**47.13 E**	**0.76 B**	**1.26 E**	**7.20 C**
Astragalus oocephalus Boiss subsp. *stachyophorus*	Before flowering	32.79a	24.63n	35.62p	0.70ef	1.71ef	8.68cd
	Flowering	26.64b	28.91kl	39.76no	0.56i-l	1.40g-j	6.64h-k
	Bear fruit	22.28de	37.99fg	49.53i	0.32r	1.15jkl	5.58l-o
	Average	**27.24 A**	**30.51 F**	**41.64 H**	**0.53 G**	**1.42 D**	**6.96 C**
Astragalus amblolepis	Before flowering	16.18nop	26.94m	58.79d	0.8cd	2.21bc	4.28qrs
	Flowering	14.55qr	32.87ij	62.06b	0.56i-m	1.7ef	3.14t
	Bear fruit	11.65s	35.86h	66.08a	0.37qr	1.23i-k	3.36st
	Average	**14.13 K**	**31.89 E**	**62.31 A**	**0.58 E**	**1.71 B**	**3.60 F**

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Astragalus declinatus Wild	Before flowering	22.53de	27.08m	36.2p	0.67fg	3.43a	11.54a
	Flowering	20.57f	29.44k	40.37no	0.57i-l	1.48f-i	10.15b
	Bear fruit	17.41klm	33.46ij	47.81j	0.52l-o	0.90lmn	9.14c
	Average	**20.17 E**	**30.00 G**	**41.46 H**	**0.58 E**	**1.94 A**	**10.28 A**
Astragalus lineatus Lam. var. *lineatus*	Before flowering	22.55de	35.73h	45.72k	0.76cde	2.21bc	7.34f-i
	Flowering	20.24fg	43.06d	50.37hi	0.65fgh	1.22ijk	7.14f-j
	Bear fruit	18.5h-k	45.6c	52.17fg	0.52l-o	0.83nop	6.58h-l
	Average	**20.43 D**	**41.46 B**	**49.42 D**	**0.65 D**	**1.42 D**	**7.02 C**
Astragalus longifolius Lam.	Before flowering	26.43b	28.74kl	36.71p	1.00a	1.82de	7.7d-g
	Flowering	23.27d	35.84h	43.60l	0.75de	0.74nop	6.98f-k
	Bear fruit	19.3ghi	48.54a	52.17fg	0.59h-k	0.59pq	6.04k-n
	Average	**23.00 B**	**37.70 C**	**44.16 F**	**0.78 A**	**1.05 F**	**6.91 C**
Astragalus aureus Willd.	Before flowering	18.61hij	24.56n	52.00fg	0.83bc	1.52fg	5.12n-q
	Flowering	16.62mno	28.42kl	57.28e	0.47no	1.11klm	4.52pqr
	Bear fruit	15.34pq	38.42f	60.64c	0.35qr	0.40q	4.14q-t
	Average	**16.85 J**	**30.47 F**	**56.64 C**	**0.55 F**	**1.01 F**	**4.59 E**
Astragalus onobrychis	Before flowering	22.56de	16.22r	45.32k	0.75de	1.25h-k	7.45fgh
	Flowering	20.07fg	18.31q	47.14j	0.63ghi	0.63opq	7.05f-j
	Bear fruit	15.97op	20.53p	49.58i	0.58h-l	0.43q	6.17j-m
	Average	**19.54 G**	**18.35 J**	**47.32 E**	**0.65 D**	**0.77 H**	**6.89 C**

CP: crude protein (%); **ADF:** acid detergent fiber (%); **NDF:** Neutral detergent fiber (%); **CT:** condense tannin (%); **CO:** crude oil (%); **CA:** crude ash (%);small letters show significant differences between the interactions of *Astragalus* species and harvest stage; capital letters show significant differences between the means of *Astragalus* species

Gas and methane production, metabolic energy and organic matter digestibility

Average gas production, methane production, metabolic energy and organic matter digestibility of *Astragalus* species harvested at different vegetation stages are provided in *Table 2*. The effects of *Astragalus* species, harvest times and *Astragalus* x harvest time interaction on gas and methane production, ME and OMD levels were found to be highly significant ($P \leq 0.01$). Gas and methane production, ME and OMD decreased with the progress of harvest time. Gas production values varied between 31.50-50.00 ml, methane productions between 3.31-6.54 ml, metabolic energy contents between 7.19-10.43 MJ kg^{-1} DM and organic matter digestibility levels between 50.47-69.52%.

Table 2. *Gas and methane production, metabolic energy and organic matter digestibility of Astragalus species harvested at different stages*

***Astragalus* species**	**Stages**	**GP**	**CH_4**	**ME**	**OMD**
Astragalus gummifer Labill.	Before flowering	46.50 cde	6.00 b	9.98 d	66.40 bc
	Flowering	39.00 jk	4.69 f	8.64 j	58.65 j
	Bear fruit	34.00 op	3.78 i	7.48 op	51.78 qr
	Average	**39.83 D**	**4.82 EF**	**8.70 EF**	**58.94 EF**

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Astragalus cinereus Willd.	Before flowering	41.50 hi	5.41 cd	9.58 e	63.50 d-g
	Flowering	37.00 k-n	4.28 g	8.62 jk	58.35 jk
	Bear fruit	33.00 pq	3.64 I	7.76 no	53.62 opq
	Average	**37.17 F**	**4.44 G**	**8.65 F**	**58.49 F**
Astragalus compactus Lam	Before flowering	50.00 a	6.22 ab	10.32 ab	69.52 a
	Flowering	45.50 def	5.30 cd	9.37 efg	63.83 def
	Bear fruit	38.00 jkl	3.76 I	8.22 lm	56.67 klm
	Average	**44.50 AB**	**5.12 BC**	**9.30 BC**	**63.34 B**
Astragalus lineatus Lam. var. *longidens*	Before flowering	49.50 ab	6.35 a	10.37 a	69.35 a
	Flowering	44.00 fg	5.11 de	9.30 e-h	63.16 e-h
	Bear fruit	37.00 k-n	4.26 g	8.19 lm	56.29 lmn
	Average	**43.50 BC**	**5.24 AB**	**9.29 BC**	**62.93 B**
Astragalus oocephalus Boiss subsp. *stachyophorus*	Before flowering	41.50 hi	5.24 cd	10.05 bcd	67.09 bc
	Flowering	39.00 jk	4.72 f	9.19 f-I	61.97 f-i
	Bear fruit	35.50 mno	3.68 I	8.34 kl	56.83 j-m
	Average	**38.67 E**	**4.55 G**	**9.19 C**	**61.97 C**
Astragalus amblolepis	Before flowering	43.00 gh	5.37 cd	9.12 ghi	60.66 i
	Flowering	39.50 ij	4.83 ef	8.42 jkl	56.75 klm
	Bear fruit	35.00 nop	3.90 hi	7.50 o	51.46 r
	Average	**39.17 DE**	**4.70 F**	**8.35 G**	**56.29 G**
Astragalus declinatus Wild	Before flowering	45.50 def	5.51 c	10.15a-d	66.22 c
	Flowering	44.00 fg	5.15 de	9.45 ef	63.91 de
	Bear fruit	39.00 jk	4.13 gh	8.42 jkl	57.98 jkl
	Average	**42.83 C**	**4.93 CD**	**9.34 B**	**62.70 BC**
Astragalus lineatus Lam. var. *lineatus*	Before flowering	48.00 abc	6.38 a	10.29 abc	68.18 ab
	Flowering	37.50 j-m	4.65 f	8.48 jkl	57.79 jkl
	Bear fruit	33.50 opq	3.86 hi	7.72 no	53.10 pq
	Average	**39.67 DE**	**4.96 D**	**8.83 DE**	**59.80 E**
Astragalus longifolius Lam.	Before flowering	47.50 bcd	5.96 b	10.43 a	69.50 q
	Flowering	44.50 efg	4.69 f	9.59 e	65.37 cd
	Bear fruit	42.50 gh	4.32 g	8.99 I	61.74 ghi
	Average	**44.83 A**	**4.99 DC**	**9.67 A**	**65.54 A**
Astragalus aureus Willd.	Before flowering	49.00 ab	6.54 a	10.01 cd	67.15 bc
	Flowering	43.50 fgh	5.49 c	9.03 hi	61.32 hi
	Bear fruit	36.50 lmn	3.74 I	7.83 n	54.50 nop
	Average	**43.00 C**	**5.26 A**	**8.96 D**	**60.99 D**
Astragalus onobrychis	Before flowering	41.50 hi	5.13 de	9.21 f-i	62.41 e-i
	Flowering	35.00 nop	4.23 g	8.01 mn	55.48 mno
	Bear fruit	31.50 q	3.31 j	7.19 p	50.47 r
	Average	**36.00 G**	**4.22 H**	**8.14 H**	**56.12 G**

GP: gas production (ml); **CH_4:** methane (ml); **ME:** metabolic energy (MJ kg^{-1} DM); **OMD:** organic matter digestibility (%); small letters show significant differences between the interactions of *Astragalus* species and harvest stage; capital letters show significant differences between the means of *Astragalus* species

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Biplot analysis

Biplot analysis revealed that two principle components were able to explain 73.10% of total variation (41.80% by PC1 and 31.30% by PC2). As can be seen in *Figure 1*; crude ash, crude protein and condensed tannin parameters constituted one group; ME and OMD constituted one group and GP, methane and ADF constituted another group. Crude oil and NDF alone separately constituted different groups. *Astragalus longifolius* was prominent with ME and OMD, *Astragalus amblolepis* with NDF, *Astragalus oocephalus* with crude protein, *Astragalus compactus* with ADF and *Astragalus aureus* was prominent with methane production *(Table1, Table 2, Figure 1).*

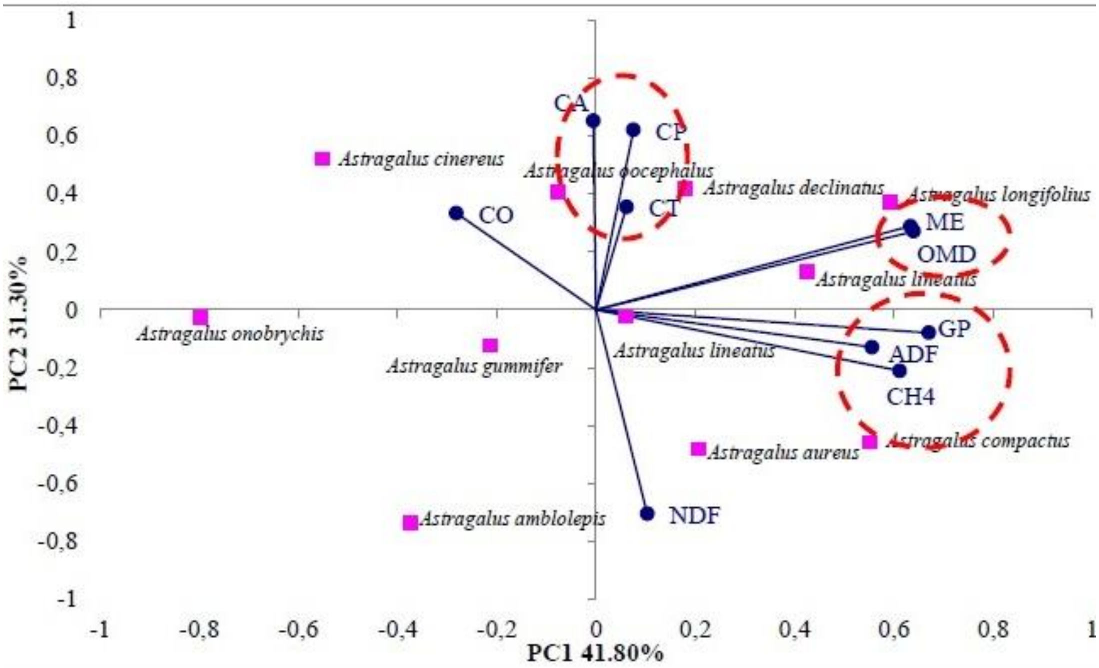

Figure 1. *The biplot for nutritive values of Astragalus species*

Discussion

Crude protein content is a significant indicator of feed quality (Assefa and Ledin, 2001). It was reported that differences in crude protein contents of different varieties can be resulted from genetics of the plants and such values can also vary depending on leaf, spike and stem ratios, ripening periods, fertilization, climate and soil conditions (Ball et al., 2011). Number of leaves and thus leaf/stalk ratios decrease with the progress of ripening. Ripening reduces number of leaves rich in crude protein and reduces crude protein ratios of the plants (Buxton, 1996). In present study, crude protein ratios of *Astragalus* species decrease with the progress of ripening and quite significant differences were observed among the species. The crude protein contents of the present study ranged within the values reported by Davis (1982) for 33 different *Astragalus* species. However, crude protein content (32.79%) of endemic *Astragalus oocephalus* at before flowering period was higher than those reported values.

The differences in stem and leaf ratios in plants result in differences also in crude protein contents, ADF and NDF ratios. ADF and NDF levels, which are the cell wall components, increased with the progress of ripening. It was reported for several plant

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species that increased ADF and NDF ratios reduced crude protein, crude oil and carbohydrate levels of the plants with the progress of vegetation period (Canbolat, 2012; Kaplan et al., 2014a, 2014b).

It was reported that low condensed tannin levels (2-3%) had beneficial effects because they prevent extreme decomposition of proteins in rumen (Barry, 1987). Kumar and Singh (1984) reported that high condensed tannin levels had harmful effects because they decrease protein digestibility. In the current study, condensed tannin levels (0.32-1.00%) were lower than those specified ones and thus such levels had beneficial effects. Crude ash is the residual unburnt portion after ashing dry matter in an as oven (Genctan, 1998). Since it cannot be synthesized by animal organisms, they should be taken externally.

Gas production of *Astragalus* species were different because of the differences in crude protein, crude oil, ADF and NDF ratios resulted from differences in their stem and leaf ratios. The increased crude oil and crude protein levels caused an increase in the metabolic energy. In vitro gas productions through fermentation in feeds are directly related to the fermentable carbohydrate content. The differences in ME and OMD levels of *Astragalus* species were mainly resulted from differences in their gas production, crude protein, crude ash and crude oil contents as well as harvest period-induced reductions in these parameters. Decreased fermentable carbohydrate contents with the progress of ripening and differences in stem and leaf ratios also decreased in vitro gas production levels (Blummel and Orskov, 1993). The gas production and thus carbohydrate levels decrease with the progress of ripening.

The feeds are classified according to the methane content after fermentation as low anti-methanogenic (> 11% and ≤ 14%), medium anti-methanogenic (> 6% and < 11%) and high anti-methanogenic (> 0% and < 6%) (Lopez et al., 2010). Accordingly, *Astragalus* species of the present study were classified as high anti-methanogenic.

Conclusion

It was concluded based on present findings that nutritive values of *Astragalus* species were quite different from each other and the progress of growing stages affected the nutritive profile of *Astragalus* samples significantly. Just because of high protein and ME contents, it was recommended that *Astragalus* species should be grazed in before flowering or full flowering stages. Among 11 *Astragalus* species investigated in this study, the endemic *Astragalus oocephalus* and *Astragalus longifolius* were found to be prominent with their high crude protein content and ME levels and low ADF and NDF ratios. It was observed that *Astragalus* species had superior properties for the animals. Further research is recommended on nutritive values of different *Astragalus* species with new in vivo and in vitro studies and also on the effects of *Astragalus* species on animal health.

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ECOLOGICAL EFFECTS OF THE FIRST DAM ON YANGTZE MAIN STREAM AND FUTURE CONSERVATION RECOMMENDATIONS: A REVIEW OF THE PAST 60 YEARS

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Abstract. The Gezhouba Dam was the first and lowermost dam on the major stem of the Yangtze River. Up to now, the dam has been operating for more than 35 years. The time period was a fast economic development stage in the Yangtze basin. Therefore, the entire Yangtze aquatic ecosystem has been highly affected by various anthropogenic activities. Especially, the fish population and distribution in the Yangtze River have been largely altered. This study reviews the ecological effects of the Gezhouba Dam to the Yangtze aquatic biodiversity for the past 60 years based on literatures. It was concluded that the pre-assessment of the Gezhouba Dam on Yangtze fishes in 1970s was appropriate. Blocking the migration of migratory fishes, such as Chinese sturgeon *Acipenser sinensis*, and the four major Chinese carps, was a critical adverse effect. Currently, the Yangtze aquatic biodiversity is facing further problems not only the existence of the Gezhouba Dam but also anthropogenic activities. Effective and practical protected measures are recommended and should be conducted urgently to rescue the Yangtze aquatic biodiversity.
Keywords: *ecological impact assessment, anadromous fish, movement and migration, Gezhouba Dam, Yangtze River*

Introduction

The Yangtze River originates from the Qinghai-Tibet Plateau and connects to the west Pacific. It is the third largest river in the world with more than 6,300 km in total length and 180 km^2 basin area which corresponds approximately one fifth of China mainland size (Yu and Lu, 2005). It has 437 tributaries with larger than 1,000 km^2 of sub-basin area and 22 of them larger than 10,000 km^2. The total area of freshwater lakes is larger than 22,000 km^2. The annual run off is 960×10^9 m^3/s. Due to the huge size, the river holds high aquatic biodiversity including about 2,000 aquatic species, thereinto there are approximate 400 fish species (Yang et al., 2007). In the world, it is the only river which holds Acipenseriformes (Chinese sturgeon *Acipenser sinensis*, Yangtze (Dabry's) sturgeon *A. dabryanus*, and Chinese paddlefish *Psephurus gladius*) and Cetacea (Yangtze River dolphin *Lipotes vexillifer*, Finless porpoise *Neophocaena phoconoides*).

Due to high elevation variations and abundant runoff, the Yangtze River contains rich water energy. The theoretical reserve of hydraulic resources is 2.78×10^8 kW, and

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the annual power generation is 2.43×10^{13} kWh (Yang et al., 2007). No hydropower dam on the Yangtze main stem was built until late 1960s. In 1970, the Gezhouba Dam was begun to construct and it blocked the river stream in 1981. It was the first and up to now the lowermost dam in the Yangtze main stem. It should be mentioned that at that time, as the economic difficulties and thought that a fish passage might not have worked effectively, a fish passage was decided not to be built in the end after countrywide discussions and disputes (Chang et al., 1998).

For 35 years, the Gezhouba Dam has been operating and affecting Yangtze aquatic organisms in various aspects. It is speculated that the ecological effects of the dam should have already emerged. Therefore, it should be the right time to review all relevant studies on the first and lowermost dam on the Yangtze main stream. More precisely, a comprehensive review on ecological impacts on the Yangtze aquatic biodiversity on the basis of the existence of the Gezhouba Dam is needed. In this study, we review the ecological impacts of the Gezhouba Dam and the relevant Yangtze aquatic biodiversity for the past 60 years mainly based on literature studies. Furthermore, this study is to re-evaluate whether the original decisions of the Gezhouba Dam's effects on Yangtze aquatic organisms are appropriate, and to examine how the mitigated protection measures recommended were performed. In addition, the future conservation strategies and measures are proposed and discussed on the basis of the current status of Yangtze aquatic biodiversity and threatening factors.

The Yangtze aquatic biodiversity

In accordance with the first systematic investigation on Yangtze fishes conducted about 60 years ago, approximately 2,000 aquatic species have been reported in the Yangtze Basin (*Table 1*) (Yang et al., 2007). According to the China Red Book of Endangered Animals, the number of threatened fishes was 69 species (occupied 18.25% of the entire Chinese endangered fishes), amphibian was 69 species (47.59%), and reptile was 54 species (32.53%). All had the highest threatened ratio among the China inland water areas. In addition, 162 fish species are regarded as endemic species. Many of them are listed as important protected species both inside and outside China (*Table 2*).

Table 1. *Major aquatic organisms in the Yangtze River system (Yang et al., 2007)*

Category	Taxon	Species numbers
Algae	Planktonic algae	321
	Attached algae	>100
Aquatic vascular plant	Fern	6
	Dicotyledon	89
	Monocotyledon	119
Zooplankton	Protozoa	93
	Rotifer	118
	Cladows	74
	Copepods	45
Zoobenthos	Oral gills	73
	Gastropods	56
	Oligochaeta	13
	Polychaeta	8

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	Aquatic insect	60
	Crustacea	7
Nekton	Fish	378*
	Mammalia	3
Amphibian and reptile	Caudata	23
	Anura	122
	Chelonia	18
	Squamata	147
	Crocodilia	1
Waterfowl		129 (Poyang lake)
Total		>1878**

* Includes 16 subspecies. ** Not includes the waterfowl and attached algae.

***Table 2.** Protected species in the Yangtze Basin*

No.	Chinese name	English name	Latin name	Protection level in China	IUCN	CITES
1	白鱀豚	Yangtze River dolphin	*Lipotes vexillifer*	I	CR	I
2	白鲟	Chinese paddlefish	*Psephurus gladius*	I	CR	II
3	中华鲟	Chinese sturgeon	*Acipenser sinensis*	I	CR	II
4	达氏鲟	Dabry's sturgeon	*Acipenser dabryanus*	I	CR	II
5	扬子鳄	Chinese alligator	*Alligator sinensis*	I	CR	I
6	江豚	Finless porpoise	*Neophocaena phocaenoides*	II	VU	I
7	胭脂鱼	Chinese sucker	*Myxocyprinus asiaticus*	II	/	/
8	金线鲃	Golden line fish	*Sinocyclocheilus grahami*	II	CR	/
9	大鲵	Chinese giant salamander	*Andrias davidianus*	II	CR	I
10	川陕哲罗鲑	Sichuan taimen	*Hucho bleekeri*	II	CR	/
11	秦岭细鳞鲑	Qinling lenok	*Brachymystax lenok tsinlingensis*	II	/	/
12	松江鲈	Rough-skin sculpin	*Trachidermus fasciatus*	II	/	/
13	花鳗鲡	Giant mottled eel	*Anguilla marmorata*	II	LC	/

Note: IUCN means the International Union for Conservation of Nature, CR (Critically Endangered), VU (Vulnerable) and LC (Least concern) are Red List Categories. CITES indicates the Convention on International Trade in Endangered Species of Wild Flora and Fauna, I or II means in Appendix I or II, respectively.

The Gezhouba Dam and reservoir

The Gezhouba Dam was located about 1,678 river km from the Yangtze estuary (river km 0 was at the estuary, *Fig. 1*). The Gezhouba Dam was constructed from 1970 for 20 years, blocked in 1981, and completely finished in 1990 (Anonymous, 1993;

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CWRC, 2002). It was the largest dam since the P. R. China constructed in 1949. The dam was 2606.5 m in length, 53.8 m in height, and 70 m in elevation. The annual flow discharge at the dam was 14,300 m^3/s, and sediment content was 1.19 kg/m^3. The reservoir capacity was 1.58×10^{10} m^3, and adjustable capacity was 0.8×10^9 m^3. The total installed capacity of the power plant was 2,715 MW. The normal water level of the reservoir was 66 m. In flooded season, the length of backwater was approximately 110 km (to Badong, Hubei Province), and in dry season, the length can be up to 180 km (to Fengjie, Chongqing Municipality). Additionally, the design of the Gezhouba Dam helped in building the Three Gorges Dam, which is the biggest dam in the world, as an anti-regulated shipping cascaded dam in 1994.

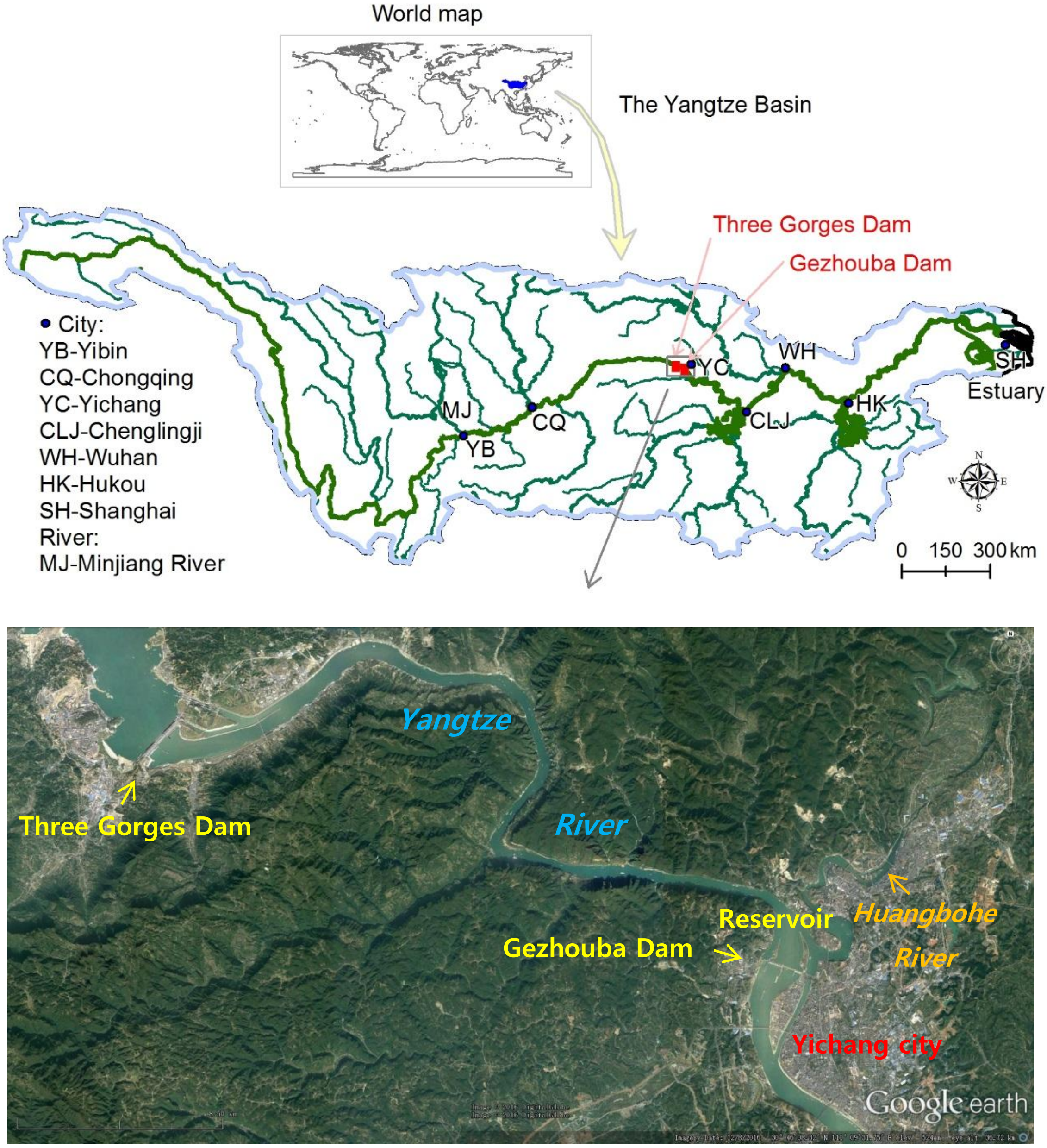

Figure 1. *The Gezhouba Dam and reservoir in the Yangtze River Basin*

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Impact assessment before the dam construction

Before the dam was constructed, a series of special studies on fishes and fisheries in the Yangtze Basin had been conducted (FLIHHP, 1976; YARSG, 1988; Yi et al., 1988; Chen et al., 1997). The studies can be summarized into three categories: field surveys, laboratory studies, and theoretical studies. The field surveys included survey on spawning areas of the four major Chinese carps, ecological survey of Chinese sturgeon, and survey on the aquatic organisms in the upper Yangtze main stem. The laboratory studies included fishes, for example Chinese sturgeon, swimming ability for designing a fish passage, and artificial propagation of Chinese sturgeon. The theoretical studies included reviews on fish passages all over the world and plans on fish rescue measures.

These studies concluded that Yichang reach had more than 100 fish species, which was equivalent to approximately one third of the entire Yangtze River fish species. As the Gezhouba Dam was a low-head runoff hydropower station, it might not have changed the hydrological regime and river fluvial habitat significantly. However, blocking the migration route of migratory fishes was the major adverse effect. On the issue on building a fish passage for free migration, national-wide and long-term disputes were aroused. This dispute finally terminated in 1982, when the spawning activity of Chinese sturgeon was confirmed below the Gezhouba Dam (Yu et al., 1985; Chang et al., 1998). Before the dam was closed in 1981, the major conclusions and recommended protection measures on migratory fishes were as follows:

1) The Chinese sturgeon: a fish passage might not have functioned properly for this species, because the species is huge (250−400 cm in length). Even though the species could swim up to the upstream by passing through the passage, a way how they could swim downstream was problematic. On the other hand, if the species might have the ability to adapt to the new environment below the dam, they probably could spawn in the new site. In addition, artificial propagation and releasing could be good methods to increase the population of the species.

2) The four major Chinese carps: as their spawning areas were widely distributed in the entire Yangtze main stem, the dam construction could only destruct the spawning areas near the dam. Blocked population would have spawning in above or below the dam respectively. Therefore, the dam would have had a little adverse effects on them and no further protection was needed.

3) Other freshwater migratory fishes: white bream *Parabramis pekinensis*, black bream *Megalobrama skolkovii*, bronze gudgeon *Coreius heterodon*, and *Rhinogobio typus* and other species would have migrated among river main stream, tributaries or lakes. Thus, they should have adapted to the new dammed river environment like the four major Chinese carps.

4) Other river-sea migratory fishes: most of Reeves shad *Macrura reevesii* and Japanese eel *Anguilla japonica* could not have swum upstream to the dam location, it was speculated that the dam would not have affected obvious adverse effects on them.

Co-effect of other anthropogenic activities

The Yangtze Basin was one of the most developing zones in China, and it produced approximately 40% of country’s Gross Domestic Product (Yang et al., 2007). Before and after the Gezhouba Dam was constructed, anthropogenic activities imposed various adverse effects on the aquatic ecosystem. The anthropogenic activities included water conservancy projects (other dams), fishing, water pollution, disconnection of river and

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lakes, sand and gravel excavation, navigation, port construction, channelization, bank protection projects, invasion of exotic species (Yang et al., 2007). Fish capture production shows that the overfishing have been occurred since early 1950s (*Fig. 2*) (SGFRYR, 1990). *Fig. 3* shows that the ship cargo volume increased by double in 1980s since the Gezhouba was constructed (Yin et al., 2002; CTGC, 2015). The increase of ship cargo volume implied the increase of ship number, ship size as well as heavy human activities on the water. These were potential threats to Yangtze aquatic biodiversity besides the Gezhouba Dam.

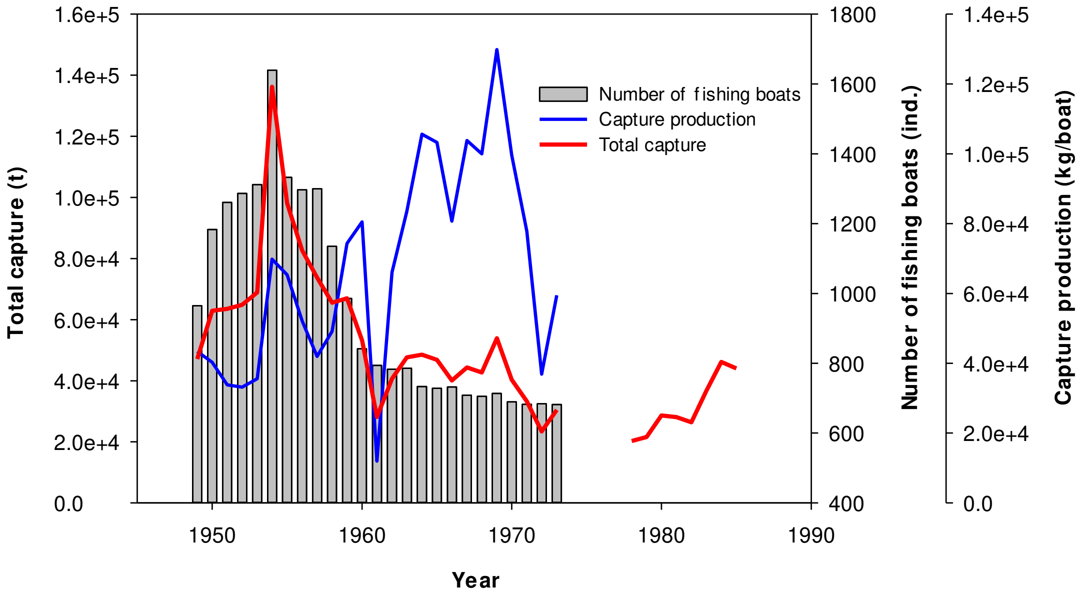

Figure 2. *Fish capture production in Hubei Province (from Yichang to Hukou) before and after the construction of the Gezhouba Dam (SGFRYR, 1990)*

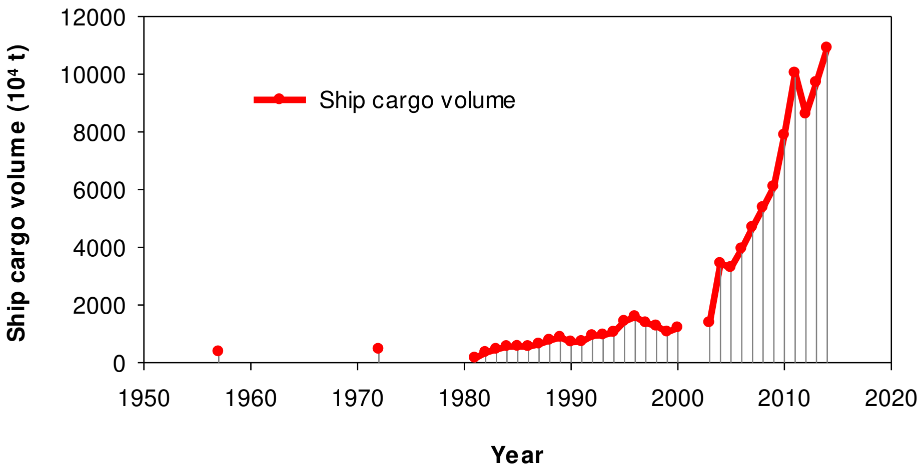

Figure 3. *The ship cargo volume before and after the construction of the Gezhouba Dam. Few data were obtained before 1980, but it was known the data in 1957 and 1972 were historical peaks because of political reasons (Yin et al., 2002; CTGC, 2015)*

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Main ecological effects of the Gezhouba Dam

Influence pathways

The main ecological effects of the Gezhouba Dam contain two aspects: 1) blocking the movement and migration of aquatic organisms directly, and 2) reservoir operation altered the river environment and then affected the aquatic organisms indirectly. *Fig. 4* presents the detailed influence pathways of the Gezhouba Dam on aquatic organisms.

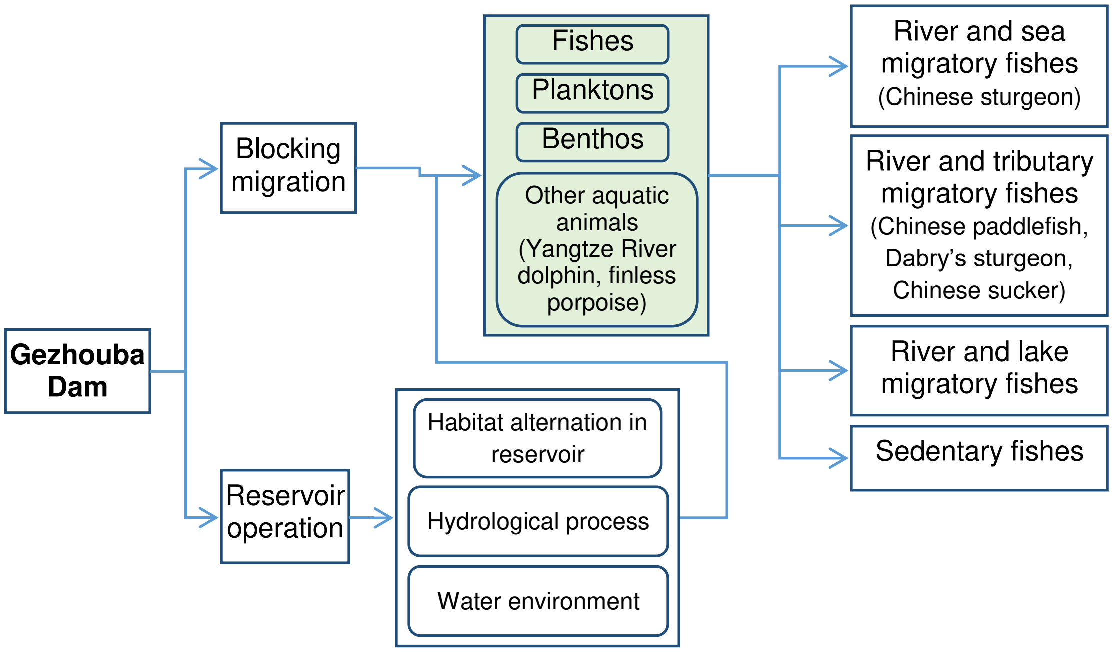

Figure 4. *Ecological influence pathways of the Gezhouba Dam on Yangtze aquatic organisms (representative endangered species are showed in the brackets)*

Effects on fishes

Effects on fishery composition

In both time periods between before and after the dam was constructed, the compositions of the fish fauna around the Yichang River section (above and below the Gezhouba Dam) were dominated by riverine and plain fish species. Hundred fish species were found before the dam closed while 94 species were observed after that closed on the basis of the investigations (WCPBHB, 1975; Wu et al., 1988). In the time after the dam was built, the proportion of fluvial fish in the reservoir area was slightly reduced, six fluvial fish species, for instance *folifer brevifilis,* were not found in the later investigation (Wu et al., 1988). As the river environment below the dam had little change compared with that before the dam was constructed, the main commercial fishery species had not fundamentally changed. The majority of fish family such as Cyprinidae and Bagridae has been observed before the dam was built, and later the dam was constructed, Siluridae was founded (Wu et al., 1988). As a result of the dam's blocking, the number of migratory fishes increased below the dam, such as the Chinese sucker. The proportion of the major economic fishes in the catch composition varied slightly, more precisely nine species such as *leiocassis longirostris* were increased.

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However, the fishery resources in the Yangtze River occurred decline trend at that time. Overall, the dam had no obvious effects on the harvest of fish production in the Yichang section (Wu et al., 1988).

Effects on river and sea migratory fishes

Three typical fish species belong to this category: the Chinese sturgeon and Reeves shad are anadromous, and the Japanese eel is diadromous. The Chinese sturgeon has been greatly affected by the Gezhouba Dam (Wei et al., 1997). Firstly, its migration route was completely blocked, approximate 1,100 km of migration route in the upper Yangtze River was not accessible. Accordingly, the spawning areas originally in that reach could not function at all. Although new spawning activities have been documented below the dam since 1982 (Chang et al., 1998), the number of spawning areas and spawning scale were significantly decreased compared with those before constructing the dam. More than 16 spawning areas had been reported in the 600 km historical spawning reach but only 2−3 new spawning areas were reported in the 30 km reach below the dam (Zhang et al., 2009). Moreover, the spawning scale was greatly decreased. Secondly, blocking made many Chinese sturgeon aggregated below the dam and over exploited by fishing. In 1981−1982, fishing on the species was not prohibited, and approximately 1,805 adults were captured which made the natural population of the species incurred a great loss (Wei et al., 1997). Thirdly, the adult population below the dam was significantly deteriorated due to the altered inhabiting environments. For instance, the sex ratio (female to male) was used to be 1:1 before constructing the dam, however it gradually became 5.86:1 in 2003−2004, which probably caused a very low spawning success rate (Wei et al., 2005). Moreover, approximately 40% adults appeared difficulties in gonadal development (Wei et al., 1997; Xiao et al., 2006). Clearly, the breeding population was ageing and the number of recruitment adult individuals became very small (Wei, 2003; Wei et al., 2005). Fourthly, as the migration route of the adults was greatly shortened, that is approximately 1,100 km, correspondingly, the living area for the filial generation of the species was considerably reduced by about 40% (Zhang et al., 2012).

On the other hand, historically, only a few of Reeves shad and Japanese eel had been found above Yichang. It was speculated that only a few of them would have migrated into the reach above the dam site. Based on the variations of capture on the two species since 1980s, the two were more affected by other anthropogenic activities such as overfishing and destruction of spawning areas. Hence, it was concluded that the Gezhouba Dam had little adverse effects on these two species (FLIHHP, 1976; SGFRYR, 1990).

Effects on river and tributary migratory fishes

The typical species which belong to this category include Chinese paddlefish, Dabry's sturgeon, Chinese sucker, brass gudgeon *Coreius heterokon*, and largemouth bronze gudgeon *C. guichenoti*. In fact, the life history of Chinese paddlefish and Dabry's sturgeon had not been fully understood (YARSG, 1988; Zhang et al., 2009, 2011). In general, they were regarded to be migrated to the Yangtze main stream (from upstream to estuary) and the tributaries. Their spawning areas were speculated to be located in the upstream near Yibin. After blocking the Gezhouba Dam in 1981, the two species were captured below the dam (Wei et al., 1997). In earlier 1980s, approximate

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10−30 paddlefish in every year were caught according to the capture records. However, the catch record decreased less than 10 individuals in earlier 1990s. Since 1995, no capture record was found. The capture record on Dabry' sturgeon showed the similar to that of the paddlefish (Wei et al., 1997). It was concluded that the number of two species has been extremely affected by the Gezhouba Dam.

The Minjiang River, a tributary in the upper Yangtze reach, was well-known as the spawning area of Chinese sucker. The construction of the Gezhouba Dam blocked its migration. Five new spawning areas for the Chinese sucker were reported below the dam in 1980s (Liu et al., 1992). However, since the 1990s, the natural spawning activity of the species was not documented in the entire Yangtze Basin. On the basis of the incidental capture records of adults, the natural population of the fish was notably declined. The Gezhouba Dam was considered to be one of the most important factors for the decline of this species.

The external morphology and migration behavior between Brass gudgeon and largemouth bronze gudgeon are very close. However, the spawning area of Brass gudgeon was widely distributed in the entire Yangtze main stream, while that of large mouth bronze gudgeon was only in the lower Jinsha River. The spawning area of Brass gudgeon was not largely changed although the Gezhouba Dam blocked its migration route (Liu et al., 1990). However, the only spawning area of largemouth bronze gudgeon was found in the upper Yangtze reach, the breeding population of this species was largely blocked at below the dam. It can be assumed that the spawning activities of this fish had been greatly affected by the dam.

Effects on river and lake migratory fishes

The typical species for this category are four major Chinese carps, as well as some other economically important fish species. Taking the four carps for instance, the number of spawning area and the estimation of spawning scale were remarkably decreased since the Gezhouba Dam was closed in 1981 (*Table 3*).

Table 3. *Spawning variations of the four major Chinese carps before and after the closure of the Gezhouba Dam in the Yangtze River on January 4th 1981*

Survey time	Survey reach	Number of spawning areas (ind.)	Number of estimated eggs and larvae ($\times 10^9$)	References
1964−1965	Chongqing−Pengze (1695 km)	36	1183.7	Yi et al., 1988
1981	Chongqing−Wuxue (1520 km)	24	173	SGSAYFMCC, 1982
1986	Chongqing−Tianjiazhen (1460 km)	30	175.5	Yu et al., 1988
2015	Jingkou−Huangshi	15	11.4	YRFA, 2016

The number of spawning area decreased by 20.0−33.3%, while the spawning scale decreased approximately 16.0% (from 1964−1965 to 1981 and 1986). In consideration with the entire Yangtze River, the spawning locations were not largely alternated. However, the spawning areas around the dam were destroyed, and the spawning areas were divided above and below the dam (SGSAYFMCC, 1982; Yi et al., 1988; Yu et al., 1988). Furthermore, hereafter the Three Gorges Dam started to be operated in 2002, the

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spawning scale decreased further to 11.4 billion in 2015 (YRFA, 2016). It was concluded that the original assessment on the effects of the dam for four carps was appropriate. However, the decline of the fish natural population was far beyond the previous judgements since various and fast developing anthropogenic activities threatened the species. An example of anthropogenic activities was that some lakes turned into farming fields. Other examples could be fishing and water pollution (SGFRYR, 1990).

Effects on sedentary fishes

Except for the reservoir area, the environmental conditions of other river sections were not changed. Thus, the distribution of the species in the river sections were not largely altered. The environmental alternations of the reservoir increased lentic fishes, yet reduced lotic fishes. However, there was only little effect on all resources of the settlement fishes (Huang, 2002), such as the common carp *Cyprinus carpio* and crucian carp *Carassius auratus*.

Effects on phytoplankton

In either above or below the dam, the species composition and biomass of phytoplankton were analogous to before and after the dam was constructed. The dominated species were Bacillariophyta, Chlorophyta, and Cyanophyta. Only in the Huangbohe Reservoir, the biomass of phytoplankton was higher than that in the original natural river (Huang, 2002).

Effects on zooplankton

The protozoan was dominant species in terms of species number, however, the copepod was the most abundant species which means it had the highest biomass. The distribution and biomass of the zooplankton were similar before and after the dam was constructed, the dominant species seemed to be nearly same (Huang, 2002).

Effects on benthos

The species and biomass of benthic organisms were very related with the river ecological environments such as water depth, substrate, water flow, water quality and food supply (plankton). As the Gezhouba Dam was a low-head run-off hydropower plant, it did not greatly change the river flowing environment. This reach was in the gorges with step riversides so that there were a few riparian areas. Hence, the benthic organisms have not been extremely influenced. Only in the Reservoir, the dominated species of benthic organisms was changed into Oligochaeta (Huang, 2002).

Effects on aquatic vascular plant

The aquatic vascular plants near the dam were relatively poor conditions either before or after the dam construction since this reach was in the gorges and had a few riparian areas as well as high fluctuation of water level and high sediment content. Therefore, it was concluded that the dam had little influence on the aquatic vascular plant.

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Effects on other aquatic animals

Other aquatic animals affected by the dam could be Yangtze River dolphin, finless porpoise, and Chinese giant salamander. Their life history could be influenced by the dam. The Yangtze River dolphin was reported to be distributed from Huanglingmiao (a site at Yichang reach, approximately 30 km upstream the dam) to the Yangtze estuary, that is approximate 1800 km in river length (Chen et al., 1997). The finless porpoise was distributed in the river reach below Yichang to Yangtze estuary which is approximately 1700 km in river length (Chen et al., 1997). The Gezhouba Dam was almost at the top of the upstream living area for the Yangtze River dolphin and finless propose. Accordingly it should not have considerable adverse effects on the two aquatic animals. The Chinese giant salamander is mainly distributed in the mountainous tributaries and stream. The Gezhouba Dam was a low-head run-off hydro power plant located in the main stem, and the reservoir was relatively small, the backwater did not greatly affect the tributaries. Therefore the dam did not have significant influence on the Chinese giant salamander.

Conservation measures

Because of the construction of the Gezhouba Dam, many protective measures have been adopted to reduce the adverse effects on Yangtze fishes. The main mitigated measures included catching and moving the Chinese sturgeon to the upstream, building a Chinese sturgeon hatchery, and conducting artificial propagation and releasing. In addition, in view of increasingly adverse impacts of various anthropogenic activities on the Yangtze aquatic biodiversity, other protective measures such as natural population protection, habitat protection, fishing ban policy, and special rescue plan for flagship species have also been adopted.

Catching the sturgeons below the dam and moving them above the dam

After the closure of the dam in 1981, many Chinese sturgeons were concentrated in the 20-km reach below the dam. To help the species to distribute above the dam, a number of the species were captured and moved to the upstream area by boats or trucks (Liu and Xiao, 1993). The fixed or drifting gill nets and setlines were used to capture the species. Fishing was highly affected by water level, discharge, and water temperature. In summer, it was difficult to catch due to high water level and vast flow discharge. In winter, the species moved less than other seasons because of low temperature, thus it was not easy to catch them. The good fishing seasons were spring and autumn, especially in autumn, the sturgeons were tended to be aggregated. When the river flow reduced to 8,000−20,000 m^3/s, it was the best time for fishing operations. In the catch, the mature individuals (gonad for stage IV) were used for artificial propagation, and immature individuals (gonad for stage III) were transported to upper area from the dam or kept for culturing them. The transportation of the sturgeons was carried out for four years (Liu and Xiao, 1993). The captured brood stock was placed in a temporary canvas basket with water, and then transported by a boat or truck to the upper reservoir area. During 1982−1985, a total of 40 individuals, with male and female ratio of 1: 1.2, were transported for trial purpose. It was concluded that transporting a number of the species over the dam to "save the species" had little significance. In fact the transported population was very small which was not enough to form spawning

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activities. To enable to form a breeding population in the upstream, a large amount of fishing was required. At that time, it was perceived that a great number of catching and transporting the species required very high costs and heavy workloads. Finally, the transportation of the species was stopped since 1986 when the spawning activity of Chinese sturgeon was documented below the dam in 1982.

Hatchery construction and artificial releasing program

In 1982, the state authorities set up the Chinese sturgeon Institute in Yichang, which charged specially for the controlled reproduction and release of this endangered species (Liu and Xiao, 1993). This was another mitigated measure to compensate for the negative effects of the Gezhouba Dam which imposed on natural reproduction of the sturgeon. In addition, sturgeons were released in both protect areas located in the Yichang and Shanghai regions by relevant enterprises and research institutes (MOA, 2015). Up to now, over six million Chinese sturgeons of different sizes (mostly less than 35 cm) have been released by the relevant units. The released sites were mainly in the middle reaches of the Yangtze River, the Yangtze River Estuary, the Pearl River, and the Minjiang River as well as other waters to support the remaining population by restocking. However, although the artificial releasing has been conducted for more than 30 years, results have not been thoroughly evaluated. A reason can be the difficulty of monitoring them in a vast spatiotemporal range. In early 21 century, only a few research indicated that more than 90% of the juveniles in Yangtze estuary was originated from natural reproduction (Zhu et al., 2002; Wei, 2003; Yang et al., 2005). Both mark-recapture and molecular genetic methods showed the similar results to the above one. That means the artificial enhanced program was of limited significance and needed further improvements.

The optimistic aspect was that many Chinese sturgeons in different ages have been stored for the controlled propagation and releasing practices. Among them, a certain number of specimens have reached sexual maturity (8 years old). The mechanism of rescuing individuals suffered from accidental by-catch or injury was clearly understood. The mechanism was used to conduct artificial reproduction. Moreover, the artificial propagation technique of Chinese sturgeon was succeed since 2009 (Guo et al., 2011; Wei et al., 2013), which allows to sustain the species in complete artificial environments. It built a good foundation for natural population enhancement, as the artificial propagation and releasing can independent of natural mature individuals, which means natural population can purely and largely be supported by artificial population.

Prohibition of fishing and listed as protected animals

As a large number of Chinese sturgeon was captured after the Gezhouba Dam blocked its migration route in 1981 and 1982, a total ban on commercial fishing of the species was implemented since 1983 (MOA, 2015). In 1989, the species was listed as one of the top-level protected animals in China. In 1996, the species was registered as an Endangered (EN) species on the Red List of the International Union for Conservation of Nature (IUCN). Later in 2010, the species was upgraded as a Critical Endangered (CR) species in the Red List. In 1998, the species was also included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Prohibition of fishing and listed as an important protected species protected this species directly and remarkably.

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Critical habitat protection and protected area constructions

To protect the only known spawning area, that is below the Gezhouba Dam, and to breed cohorts of Chinese sturgeon, the "Yangtze Provincial Nature Reserve for Chinese Sturgeon" was established in 1996 in Yichang, Hubei Province (MOA, 2015). To protect the sturgeon juveniles and their feeding grounds along the Yangtze River estuary, the "Yangtze Estuary Nature Reserve for Chinese Sturgeon in Shanghai" was established in 2002. Some other protected areas were also set along the middle and lower reaches of the Yangtze River. For example, the "National Nature Reserve for Baiji in Xinluo Section of the Yangtze River in Hubei" played a positive role in the protection of this species and its habitats.

Fishing ban policy

Since 2003, the fishing ban policy was executed in the Yangtze Basin. In the Yangtze main stream from Deqin County, Yunnan Province to the Gezhouba Dam and some tributaries such as Min River, Jialing River, Wu River, and Chishui River (*Fig. 1*), fishing was forbidden from 12 o'clock of February 1 to 12 o'clock of April 30 every year. In the Yangtze main stem from the Gezhouba Dam to Yangtze River estuary, and Hanjiang River, as well as Dongting lake and Poyang lake, fishing was prohibited from 12 o'clock of April 1 to 12 o'clock of June 30 in each year. In December 23, 2015, the Ministry of Agriculture announced to enforce the fishing ban policy. The fishing ban area in the Yangtze main stem was extended into a longer stretch from Qumalai County, Qinghai Province to the estuary. Tributaries for example Tuo River, Dadu River, and Huai River were included. Moreover, the fishing period in the whole Yangtze Basin was unified into a four-month period from 0 o'clock of March 1 to 24 o'clock of June 30 every year. The fishing ban area and time period cover nearly all spawning areas and time periods of major fish species in the Yangtze River. Hence, it protected the breeding populations and their spawning activities very effectively. Below the Gezhouba Dam, the spring spawning species and their spawning activities could be well protected. However, the autumn spawning species, such as the Chinese sturgeon, was outside the fishing ban period.

Flagship species protection

In consideration with the fact that few rare and endemic species are on the verge of extinction, the Chinese government has exclusively designed protection measures on these species, such as Chinese sturgeon and Yangtze finless porpoise. Some decree documents issued to upgrade the Finless porpoise as a Class I protected animal. The central government also designed a very long period rescue plan for them, such as "The Action Plan for Conservation of the Chinese Sturgeon (2015−2030)" (MOA, 2015) and "The Action Plan for Conservation of the Yangtze finless porpoise (2016−2025)". Take the action plan for Chinese sturgeon as an example, it includes *in-situ* and *ex-situ* conservation actions, conservation action for genetic resources and supporting actions. The action plans will be used as guidelines and will play a very important role for future conservation of the two species.

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Conclusions and conservation recommendations

In summary, the original judgement regarding the ecological effects of the Gezhouba Dam on Yangtze aquatic organisms was appropriate. However, after the construction of Gezhouba Dam, the economic and social environments of the Yangtze River Basin have been undergone in great developments. The adverse effects of various anthropogenic activities on the Yangtze aquatic ecosystem have become more intensive and extensive (Zhang et al., 2016). Although some protective measures have been taken, the current aquatic biodiversity and fishery resources of the Yangtze River are still declining rapidly (Yang et al., 2007). Currently, the impacts of the Gezhouba Dam on aquatic biodiversity in the Yangtze River would be of little significance because the anthropogenic activities and ecological environment of the Yangtze Basin have been undergone in great changes. Overall, to protect the Yangtze aquatic biodiversity, it is recommended to focus on the following five aspects of work.

1) To further enhance the fishing ban or even to prohibit fishing in the entire Yangtze Basin gradually. The four-month fishing prohibition period at present is not enough to protect the fish biodiversity. It is recommended to extend the prohibition period, or to have a very long prohibition period for example ten years, in that period, most of the fishes can reproduce for 2−3 generations. The prohibition area can be extended gradually, the nature protected area and aquatic resource protected area could be the first priority, then moved to the main stem and tributaries. Lastly the lakes, such as Dongting Lake and Poyang Lake, where fishing is still important for a great number of fishermen to earn their livings. It is believed that the enhanced fishing ban below the Gezhouba Dam (80 km reach below the dam is a nature protected area in province level) will be of great importance to protect the migratory fish populations and their spawning activities.

2) To further strengthen habitat conservation and rehabilitation, the following action can be considered: the construction and management of protected areas, optimizing dam ecological dispatch, rehabilitating critical habitats and reconnecting of migratory channels.

3) To further enhance the protection of flagship species such as Chinese sturgeon and finless porpoise. The protection measures in the two action plans should be well performed in the future. It should be mentioned that with the emphasis on protection of these flagship species with extensive living space and long life-span, actually it can protect the whole Yangtze aquatic biodiversity.

4) To further strengthen the policy guidance to make artificial propagation and releasing more effectively, to control the alien species strictly to prevent biological invasion, and to revise the list of protected species.

5) Last yet not least, a watershed collaborative management mechanism is urgently needed. To enhance communication and cooperation between national and local administrative organizations along the Yangtze River, achievable and comprehensive improvement-oriented management for the basin environment should be accomplished.

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STAND LEVEL CO_2 FLUX EXAMINATION OF WEED SPECIES WITH DIFFERENT ORIGIN AND FUNCTIONAL GROUPS

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Abstract. Although the ecological attributes of many weed species are well-known, their eco-physiological relations – particularly at stand level – are scarcely researched. Based on this the main objectives of our examination are to measure the stand level CO_2 flux (NEE) for 5 weed species generally widespread in Central Europe as well as to explore the dependence of NEE on air temperature, radiation and stand level evapotranspiration. The selected weed species represent archaeophyte and neophyte plants as well as different functional groups (C_3, C_4, therophytes and cryptophytes). The research covering an entire vegetation cycle of the weed species has been implemented *ex situ* in 15 patches of the Szent István University's Botanical Garden. Stands of the involved species showed really significant CO_2 uptake for months. This value is the multiple of what is indicated in literature regarding Central European herbaceous vegetation (e.g. grasslands) – this fact can be one of the key factors leading to the success of this plant group. By comparing the average CO_2 fluxes of these five weed species in the entire vegetation period it can be concluded that the values of the two endemic species are almost completely identical exceeding the average values of non-endemic C_4 weed species. Furthermore, significant linear regression ($P<0.05$) has been observed between the net ecosystem CO_2 gas exchange of *Ambrosia artemisiifolia* and *Chenopodium album* and the values of stand level evapotranspiration.
Keywords: *canopy chambers; weeds; net ecosystem; carbondioxide exchange; dependence*

Abbrevations: *Ama ret – Amaranthus retroflexus*; *Amb art – Ambrosia artemisiifolia*; *Art vul – Artemisia vulgaris*; *Che alb – Chenopodium album*; ET – evapotranspiration; LAI – leaf area index; NEE – net ecosystem CO_2 exchange; PPFD – photosynthetically active photon flux density; *Sor hal – Sorghum halepense*; T_{air} – air temperature

Introduction

One of the main objectives of recent international ecosystem researches is to measure the cycles of important greenhouse gases (including carbon dioxide) in different habitats (e.g. Yamuki et al., 1997; MacDonald et al., 1998; Dong et al., 2000; Papen et al., 2001; Hefting et al., 2003; Horváth et al., 2006). Such European projects have examined ecosystems of grasslands, shrubs, waters, watersides and forests together with their plant communities/stands. However it is surprising that stand level research regarding greenhouse gas fluxes of weed stands has not yet been conducted although they can have fundamental influence on the carbon balance of certain areas due to their wide range and constant expansion. Ecological characteristics of several weed species are well-explored (e.g. Holzner and Numata, 2013; Čekić and Kovačević, 2015; Kleunen et al., 2015) but their stand level ecophysiological relations are hardly examined.

Examinations implemented so far on greenhouse gases in relation with weeds mainly focused on the impacts of increased CO_2 concentration on the growth and production of

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weed species instead of their CO_2 fluxes. Based on the responses given by different C_3 and C_4 weed species on increased CO_2 concentration in the air the highest growth rate among the involved taxa belonged to the C_4 *Amaranthus retroflexus*. In case of *Ambrosia* growth was more intensive under increased CO_2 concentration in the first half of the examination period while in the second half current atmospheric conditions (i.e. 350 ppm) resulted higher growth (Garbutt et al., 1990). The amount of reproductive biomass was increased in case of *Amaranthus retroflexus*, *Chenopodium album* and *Ambrosia artemisiifolia* when atmospheric CO_2 concentration was higher. Strong correlation can be observed between the change of leaf surface and accumulation of biomass in case of *Abutilon theophrasti* and *Amaranthus retroflexus* (Akerly et al., 1992). Development of leaves has been accelerated for these species by increasing either the air temperature or the CO_2 concentration.

Other researchers examined the impacts of not only increased but also decreased carbon dioxide concentration on – among others – C_3 and C_4 weed species (Dipperey et al., 1994.). Regarding C_4 taxa CO_2 concentration had no effect on either growth rate or total biomass within the first 35 days following germination. In case of C_3 taxa biomass showed positive correlation with higher atmospheric CO_2 concentration.

The effect of aridity was different in the involved functional groups (Ward et al., 1999). Photosynthetic rate of plants with good availability rate has increased when atmospheric CO_2 concentration was higher but their leaf surface and biomass has not changed. As opposed to that both leaf level CO_2 uptake and biomass of C_3 taxa increased under the concentration of 700 ppm. Due to the aridity leaf surface of C_3 plants reduced significantly; as a consequence of that water potential value of remaining leaves became relatively high. The LAI of C_4 plants decreased in smaller extent. Lower water potential value was measured in their leaves. Under non-limiting nutrient provision whole individuals of C_3 taxa developed significantly as a result of increased CO_2 concentration while more moderate growth has been examined in case of C_4 plants (Bernacchi et al., 2000). In limiting environment neither types showed significant development. Shoot-root rate remained unchained or decreased in the observed taxa as a result of higher CO_2 concentration.

Considerable competition can occur among weeds in mixed communities as a result of which the amount of biomass – both above and below soil surface – change as well as the habit of the plants (e.g. Bazzaz et al., 1989). Interference of different species can be observed not only in competition for resources. For instance, *Artemisia vulgaris* has allelopathic characteristics among the examined weed species (Barney et al., 2005). In mixed vegetation species can influence not only negatively the development of other taxa. Weeds growing under higher plants can utilise absorbed and photosynthetically active radiation with higher efficiency (Gramig et al., 2006). Selected parameters can be examined in a taxon specific way (Borjigidai et al., 2008) in monodominant stands of the selected weed species.

By examining archaeophyte and neophyte weed species representing different physiognomy and functional groups the CO_2 balance of these stands and plant communities can be explored. Photosynthetic capacity of invasive plants is usually higher (Nielsen et al., 1993; Patterson et al., 1998) but their mass invasion is not a primary consequence of this feature (Brodersen et al., 2008).

Measurement of carbon dioxide fluxes in the weed stands can also provide a basis in the future for monitoring alterations occurring as a result of global climate change regarding these stands and plant communities dominated by these species. Due to

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climate change extreme weather conditions become more frequent (Brodersen et al., 2008) to which taxa give different answers. Examining the success of adaptation to extreme precipitation conditions may help when establishing a prognosis in relation to the future range of certain weed species since different types of photosynthesis influence the water utilisation efficiency of plants (Niu et al., 2005; Sharkawy, 2009).

The main objective of our research is to implement comparative measurement of carbon dioxide fluxes at stand level in case of five weed species generally widespread in Central Europe (Lososová et al., 2006): *Amaranthus retroflexus, Ambrosia artemisiifolia, Artemisia vulgaris, Chenopodium album* and *Sorghum halepense*. Dependence of NEE on air temperature, radiation and stand level evapotranspiration has also been examined.

When selecting the species it has been taken into consideration that archaeophyte (*Artemisia vulgaris, Chenopodium album*) and neophyte (*Amaranthus retroflexus, Ambrosia artemisiifolia*, *Sorghum halepense*) plants as well as members of different functional groups /C_3 (*Ambrosia artemisiifolia, Artemisia vulgaris, Chenopodium album*), C_4 (*Amaranthus retroflexus, Sorghum halepense*); therophyte (*Amaranthus retroflexus, Ambrosia artemisiifolia, Artemisia vulgaris, Chenopodium album*), cryptophyte (*Sorghum halepense*)/ species and angiosperms (3 families and 5 genera) are all to be represented.

Materials and Methods

Site description

Examination covering the entire vegetation cycle of weed species have been implemented in the Experimental Site of Szent István Egyetem Botanical Garden (Gödöllő 19°14’E, 47°25’N, 250 m elevation) (Szirmai et al., 2014). Mean annual temperature is 10.5 °C; average amount of precipitation is 500 mm. The soil of the site is loose and moderately calcareous sand (Szente et al., 1993b). 15 homogenous stand patches have been created within the selected area. The size of the patches was 1 by 1 metre, with 3 repetitions by species and by examinations. Proper isolation distances have been kept among the stands. Parcels were established following random block pattern in order to avoid spot effect.

Stand level measurements

Measurements were conducted in the 2008 vegetation period by using CIRAS-2 infrared gas analyser (PP Systems, Hitchin, UK) and the so-called “open chamber” method. Measurements have been taken weekly or in every two weeks with the help of transparent, water-clear plexi chambers adjusted to the actual height of stands. The chamber used initially consisted of a cylinder wall with 60 cm diameter and a half-sphere shaped transparent part installed at the top airtight (in details see Czóbel et al., 2004). In order to harmonise the tools with the actual height of stands 70 cm and 200 cm high chambers were used. External fans ensured the consistent mixing of air within the chambers as well as air exchange. The same measurement technique has been applied regardless the size of the chambers. Measurements were taken between 11 am and 3 pm under cloudless and clean sky. Data recording took at least 30 minutes for each weed species and treatment. The equipment measured the difference in stand level CO_2 flux (in the incoming and outgoing tubes of the chamber) by taking 10 samples per

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minute. It also recorded the photosynthetically active radiation (PPFD), air temperature (T_{air}) and evapotranspiration (ET) in the stand covered by chamber. Average temperatures of the given stand have been used for CO_2 flux calculations. The following differential equation has been applied when calculating CO_2 flux:

$$F=\Delta c* Q/A$$

where 'F' is the stand level flux /CO_2 uptake of the plant stand, vegetation and soil respiration in case of CO_2 flux; the resultant of the evaporation regarding soil surface covered by vegetation in case of evapotranspiration/ (mol m^{-2} s^{-1}), 'Δc' represents the difference in incoming and outgoing concentration /CO_2 or H_2O/ (mol m^{-3}), 'Q' stands for flowrate (m^{-3} s^{-1}) and 'A' is the soil surface covered by the chamber (m^2).

Following quality control raw data have been manually filtered where negative evapotranspiration values as well as outliers recorded at the initiating and final phases of the measurement (due to human respiration) have not been taken into account.

Parallel to CO_2 flux measurements ET, PPFD and T_{air} have been recorded by patches with a portable infrared gas analyser /CIRAS-2 (PP Systems, Hitchin, UK)/

Statistics

Only significant correlations have been represented in the figures indicating the strength of the correlation. The strongest significant correlation has been fitted to the data in all cases. Values of regression have been calculated by SigmaPlot 8.0 software. In case of time series t-tests were implemented to check the level of significance regarding correlation values, impacts of manipulation experiments and the differences among the involved taxa. Standard deviance (SD) values are indicated in the figures.

Figures have been created by using SigmaPlot 8.0 software.

Results and Discussion

Net ecosystem CO_2 exchange (NEE) examinations

All five species reached considerable CO_2 exchange values until the time of cutting (*Fig. 1*). Significant values of standard deviance is connected to high CO_2 fluxes regardless of the species. Stands of *Ambrosia artemisiifolia* and *Sorghum halepense* reached the maximum of CO_2 uptake relatively late. Maximal value of CO_2 fixation, in case of both species, can be the multiple compared to that of either a fully developed loess grassland (Frank et al., 2002; Hunt et al., 2002; Czóbel, 2007; Czóbel et al., 2008) or a stand of spring forest geophytes (BARCZA et al. 2011). Different functional groups cannot be significantly separate based on their NEE values. During spring both early germinating C_3 species (*Che alb*: -9.20; *Art vulg*: -6.28) produced remarkably high NEE values even from May while stands of C_4 weeds showed lower CO_2 uptake (*Ama ret*: -4.06; *Sor hal*: -2.53) in this period. Average net ecosystem exchange increased in a great extent (*Ama ret*: -8.29; *Sor hal*: -8.44) during summer. Stands of *Amb art* reached the highest value of the summer period (-15.95) by showing great increase in biomass and NEE. In this season average NEE value of *Art vul* also increased (-9.98) while in stands of white goosefoot it has been reduced (-7.66). Order of CO_2 fixation among the species has not reflected the amount of biomass. Following cutting CO_2 exchange of the weed species significantly decreased as a result of both losing the majority of foliage

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and the poor regeneration due to aridity stress, therefore differences among taxa have been levelled off. During the autumn period *Ambrosia artemisiifolia* and *Sorghum halepense* showed the highest (-2.85) and the lowest (-0.85) average NEE value, respectively.

In case of *Amaranthus retroflexus* until uptake irrigated stand and no-precipitation stand showed the most intensive and lowest CO_2 uptake, respectively.

NEE averages of the examined weeds remarkably exceeded values measured in temperate grasslands (e.g. Frank et al., 2002; Hunt et al., 2002; Soussana et al., 2007). Compared to grasslands the examined weed species were characterised by high CO_2 fixation even in the arid summer period (Valentini et al., 1995; Suyker et al., 2003; Li et al., 2005; Pintér et al., 2008).

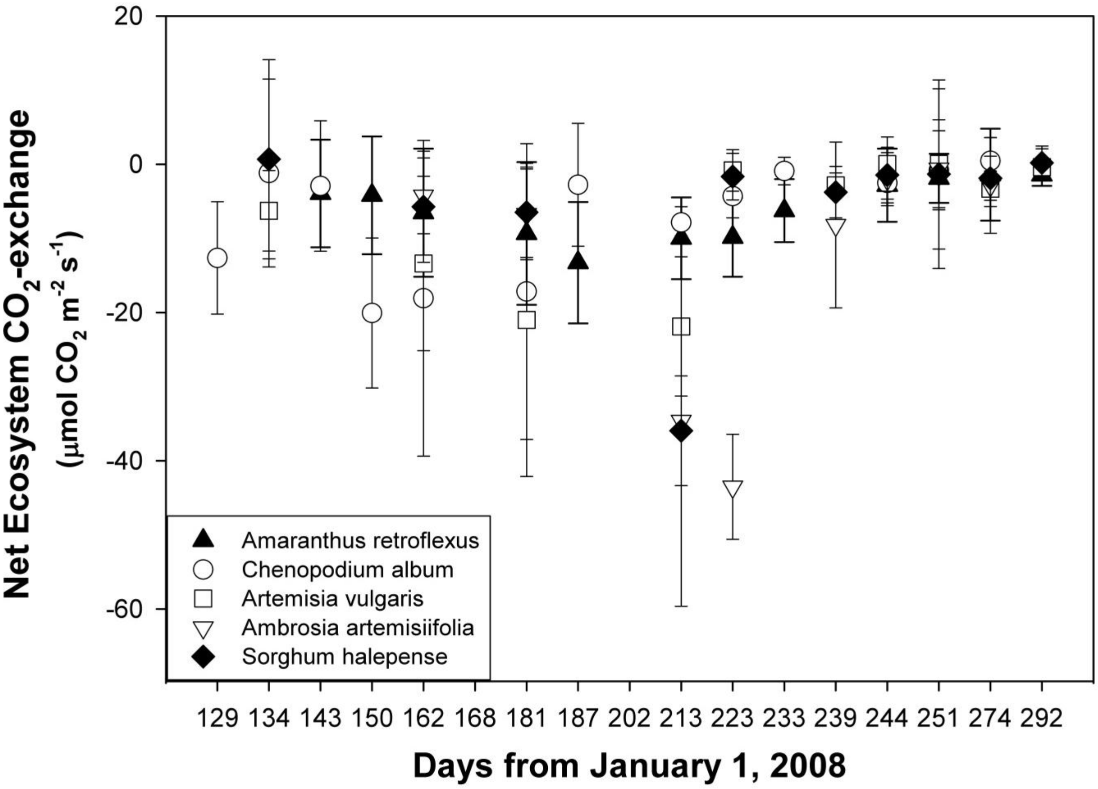

Figure 1. *Seasonal dynamics of net ecosystem CO_2 exchange (NEE) regarding the examined weed species during an entire vegetation period*

Dependence of NEE on light, temperature and evapotranspiration

As regards of NEE light dependence no significant difference could be observed among neither the 5 examined weed species nor C_3 and C_4 taxa (*Fig. 2*). Positive correlation was found between CO_2 uptake value of the stands and PPFD. In stands of *Chenopodium album* this relation was significant when using exponential fit ($P<0.05$). The most intensive CO_2 fixation was measured when PPFD value ranged between 1400 and 1600.

CO_2 fixation of the stands showed Gaussian curve when their dependence on air temperature has been examined (*Fig. 3*). No clear difference could be observed between C_4 and C_3 species. Correlation between the two parameters was significant in stands of *Chenopodium album* ($P<0.05$).

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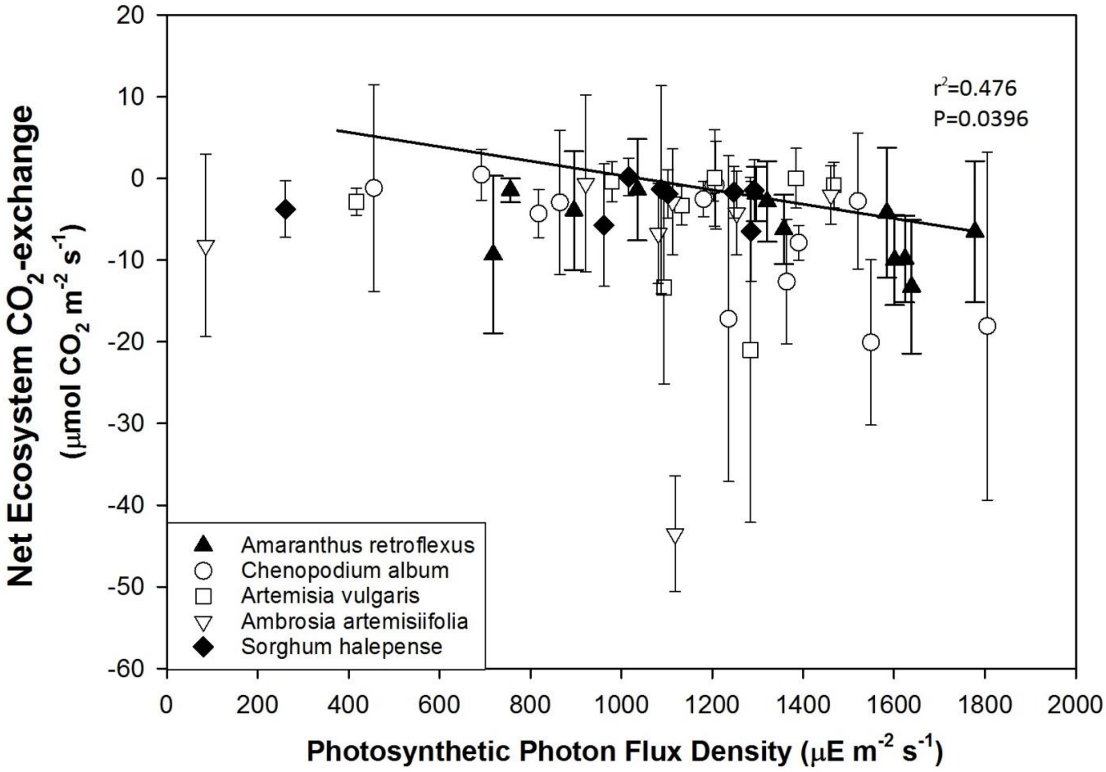

Figure 2. *Dependence of net ecosystem CO_2 exchange (NEE) regarding the examined weed species on photosynthetically active radiation (PPFD)*

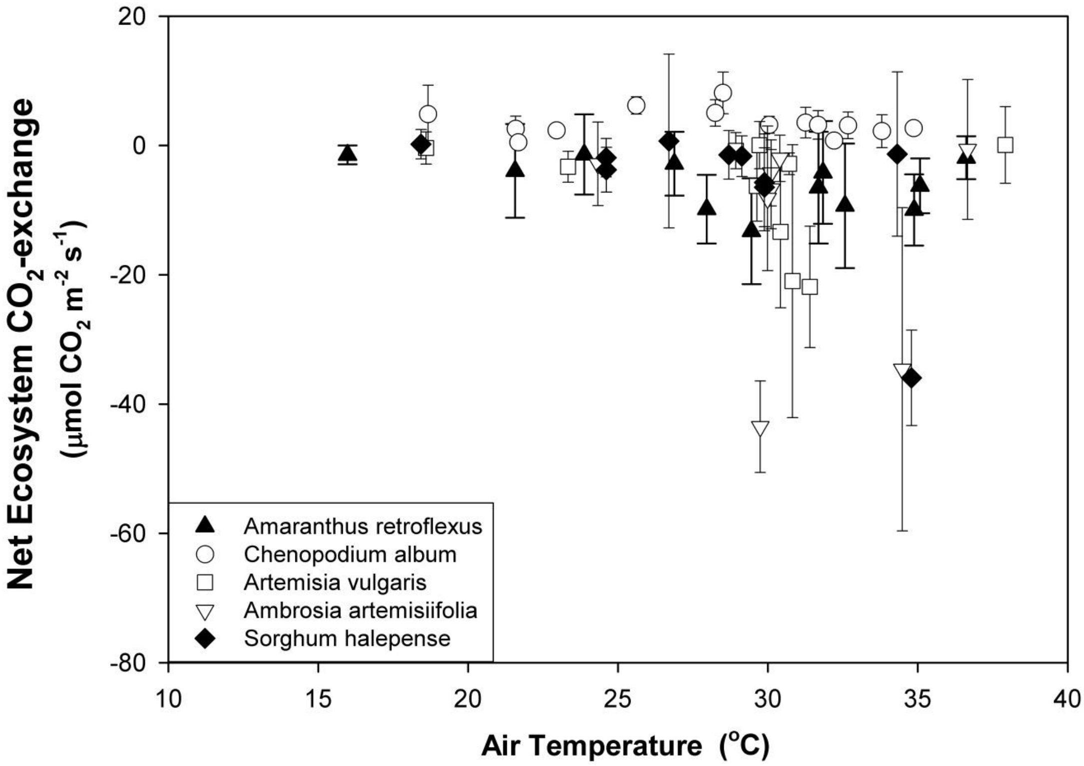

Figure 3. *Dependence of net ecosystem CO_2 exchange (NEE) regarding the examined weed species on air temperature (T_{air})*

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Considering CO_2 fixation optimum heat ranged between 28 and 35 °C for all five species. Gas exchange intensity continuously increased up to 30 °C in case of each taxon while considerable retrogression was observed over 35 °C. Latter phenomenon was experienced for C_4 type weeds also, thus presumably not only stomatal closure (C_3) but also lover level of soil humidity contributed in its development.

Positive correlation could be noticed between CO_2 uptake and ET within the examined stands (*Fig. 4*). ET dependence of NEE did not show significant distinction regarding the plant species and functional groups. However, significant linear regression ($P<0.05$) could be observed between net ecosystem CO_2 exchange and stand level evapotranspiration in case of *Ambrosia artemisiifolia* and *Chenopodium album*. When conducting the measures increased ET was usually coupled with slightly higher CO_2 uptake.

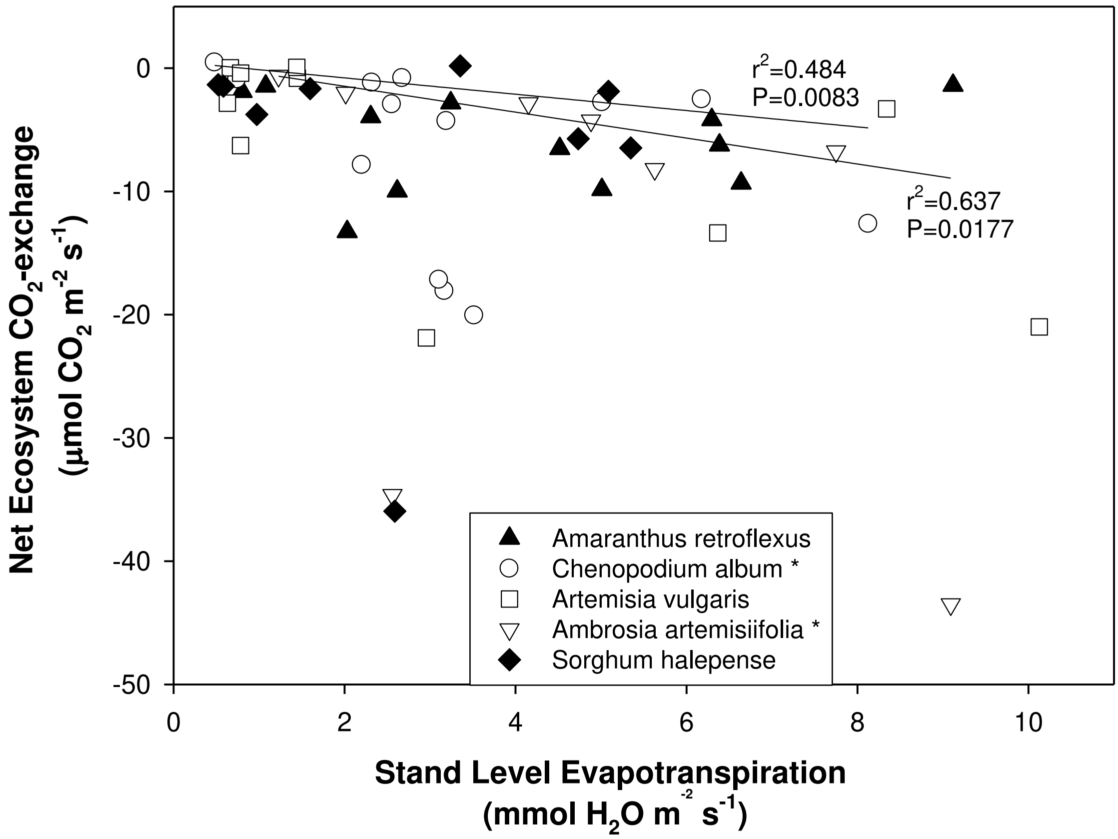

Figure 4. *Dependence of net ecosystem CO_2 exchange (NEE) regarding the examined weed species on evapotranspiration (ET)*

Conclusions

The examined species all reached remarkable production that is the multiple of values presented in literature regarding herbaceous vegetation (e.g. grasslands) in the Carpathian Basin. Despite the species represented different functional groups and characterised by different stem count and biomass they all showed very similar dynamics. This can be explained by the above-average precipitation of the examination year and by the high level of CO_2 fixation maintained for months. This latter feature can prove as one key feature leading to the success of this plant group.

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By examining the stand level CO_2 fluxes of the five involved weed species regarding the entire vegetation period it can be concluded that values of two species endemic in Central Europe (*Chenopodium album* and *Arthemisia vulgaris*) are nearly identical while exceeding the average values of non-endemic C_4 weed species (*Amaranthus retroflexus*, *Sorghum halepense*). The latter two plants, despite their different morphological features, can be characterised by almost the same average regarding CO_2 uptake. The average CO_2 fixation of the neophyte ragweed significantly exceeded that of all other plants involved. As for C_3 weeds endemic species initiated more intensive CO_2 uptake compared to the also C_3 but invasive *Ambrosia arthemisiifolia*. C_4 taxa reached higher NEE values later, similar to *Ambrosia*.

Further study is needed to define and measure the effects of intraspecific and interspecific competitions.

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RELATIONSHIP OF GERMINATION AND ESTABLISHMENT FOR TWELVE PLANT SPECIES IN RESTORED DRY GRASSLAND

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Abstract. There is a lack of knowledge on germination capabilities of native species and their relation to field establishment that could help optimize ecological restoration efforts. We studied laboratory germination and second year field establishment of 12 native vascular dry grassland plant species in the frame of a restoration project in NE Hungary. Our questions are:1) For which species is cold-stratification necessary to break dormancy of seeds? 2) Is there a positive correlation between germination and second year establishment? Laboratory germination was studied with and without cold-stratification and the impact of cold-stratification was tested by linear model. Relationships between germination and field establishment were tested by generalized linear models. Cold-stratification decreased significantly the germination of four grass species, and was important to break dormancy for one dicot. Field establishment is positively connected with germination under both germination treatment. We conclude that, laboratory germination has a high predictive value on the establishment success of seeded species.
Keywords: *cold-stratification, field plant cover, germination percentage, Hungary, sandy grassland*

Nomenclature: Scientific plant names follow the nomenclature of Király (2009).

Introduction

The unprecedented loss of biodiversity and the degradation of habitats justifies the need for ecosystem restoration, which is currently acknowledged at the policy level (Aronson and Alexander, 2013; Suding et al., 2015) and as a result, large-scale restoration efforts are planned (Aichi Target 15, SCBD, 2011). To support this volume of restoration need, the use of seeds of native species has to be scaled up and survival success has to be increased (Merritt and Dixon, 2011). Difficulty in achieving scaling up lies partly in the lack of sufficient amount of seeds and wasting by seeding more than necessary in the hope of restoration success (Williams et al., 2002; Hedberg and Kotowski, 2010; Merritt and Dixon, 2011). The other limitation of restoration is that seeding often fails to reach restoration target (Pyke et al., 2013) due to scattered knowledge on requirements of early life stages of reintroduced plant species (Larson et al., 2015). The early stages of plant recruitment are essential in the reestablishment of wild species at restoration areas as high mortality occurs during this period (James et al., 2013; Carrington, 2014). Advancements in the science of seed ecology and early life stage characteristics of plants is needed to

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underpin restoration success (Commander et al., 2009; Larson et al., 2015) and help to assess seed amount in future restoration planning.

Spontaneous succession is often hindered by the lack of seeds that originates from the depletion of soil seed bank and limited seed dispersal (Kiehl et al., 2014). Seed introduction methods to overcome this limitation include among others direct seeding, diaspore transfer with substrate, hay or brush harvesting, slot seeding, plug planting (Hedberg and Kotowski, 2010) or, under extreme conditions, e.g. in Mediterranean areas hydroseeding (Sheldon and Bradshaw, 1977; Oliveira et al., 2012). Direct seeding is the most widely used restoration method (Kiehl et al., 2010; Török et al., 2011). The composition of a seed mixture is dependent on the condition of the restoration site, the availability of seeds of target species (Török et al., 2011) or their dominance in the target vegetation (Hedberg and Kotowski, 2010). Commercial seed mixtures can be purchased easily and in large quantity. However adverse climatic and soil conditions can hinder their establishment or if established, highly competitive, fast-growing species can constrain further vegetation development (García-Palacios et al., 2010). In contrast, difficulty in using native seeds lies in the lack of seed market or scarce information on seed biology (Commander et al., 2009).

Germination is a critical transition influencing survival after seeding (Larson et al., 2015; Gallagher and Wagenius, 2016). Without knowledge on germination characteristics, the necessary amount of seeds for restoration cannot be properly estimated. Germination is generally studied under laboratory or greenhouse conditions by testing germination percentage (Grime et al., 1981; Haslgrübler et al., 2013; Larson et al., 2015; Gallagher and Wagenius, 2016). Knowledge gained during these experiments can be exploited in restoration to estimate the necessary amount of seeds; to identify best seeding time for different species based on cold-stratification requirements (Jones and Kaye, 2014), or optimal pre-treatment of seeds (Krock et al., 2016). Seed dormancy, a key attribute in the diversification of seed plants is another critical determinant of establishment success (Willis et al., 2014), and thus influences species distribution (Donohue et al., 2010). In order to enhance restoration success, breaking dormancy is a key issue. Physiological seed dormancy is the most common type of dormancy (Baskin and Baskin, 2004). Non-deep physiological seed dormancy might be broken by cold-stratification treatments (Baskin and Baskin, 2004; Krock et al., 2016), with an optimal dormancy breaking time between 2 to 12 weeks (Jones and Kaye, 2015; Kaur et al., 2016). Krock et al. (2016) investigated ten prairie species and discovered the optimal cold-stratification time below the standard protocol guidelines at eight species, they suggest 15 to 60 days of treatment. In the seed germination database of Hungary, eight of the twelve species involved in our study were pre-treated by cold-stratification of 1 week (Peti et al., 2017). There is a lack of knowledge concerning the relationship of germination ability and establishment of herbaceous plants that are most widely used in vegetation restoration (Carrington, 2014). Species must first be able to germinate to establish (Willis et al., 2014) thus producing seedlings that are prone to further mortality losses. Larson et al. (2015) have disentangled the relative importance of physiological and morphological traits that influence germination and early recruitment success for several species, but the time span of their experiment has not expanded over three weeks. Oliveira et al. (2012) found that the germination ability of single species cannot predict the behaviour in multispecies situations as co-seeding of species with various traits can have adverse results. The early seedling stage is critical under field conditions, given the stochastic nature of early recruitment events and high

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mortality in the first summer (De Steven, 1991; Oliveira et al., 2012), establishment by the end of the first growing season is an important milestone to predict restoration success (Clarke and Davison, 2004; Kiehl et al., 2014; John et al., 2016). However, little is known on later stages of seeded species. Only few studies have compared early life stage transition and second year field establishment in grassland restoration projects (Kotorová and Lepš, 1999; Clarke and Davison, 2004).

In this study, we attempt to link germination ability to second year field establishment. We analyse twelve grassland species native to the Pannonian ecoregion that are used, among others in a restoration project at a factory site in NE Hungary. The following questions are addressed: 1) For which species is cold-stratification necessary to break dormancy of seeds? 2) Is there a positive correlation between germination and second year establishment?

Materials and methods

Study area

The restoration experiment is located in NE Hungary, in the acidic inland sand dune region of Nyírség (lat 47° 57' N; long 21° 39' E). Annual average temperature is 9.8 ºC, average precipitation is 550-600 mm. The natural vegetation has been mostly transformed by human use, resulting in the loss of natural habitats and the expansion of invasive species (e.g. *Asclepias syriaca, Robinia pseudo-acacia,* Botta-Dukát (2008)). The construction of a new factory enabled us to carry out an experiment with the aim to restore native vegetation at the factory area (approx. 7 ha). Target communities are open (*Festuco vaginatae – Corynephoretum* Aszód (1935), priority habitat: 6260 Pannonic sand steppes (Romão, 1996)) and closed sandy grasslands (*Pulsatillo hungaricae - Festucetum rupicolae* Borhidi (1996)*, Potentillo arenariae – Festucetum pseudovinae* Soó (1940), priority habitat: 6260 Pannonic sand steppes (Romão, 1996)). The site was used as agricultural field, mostly apple orchard prior to the construction. The closest semi-natural dry grassland is 2.5 km far from the factory at a kurgan.

Grassland reintroduction started in 2014 in the form of seeding (with the application of mulch) or hay transfer. Two fescue species were seeded in larger quantities (*Festuca rupicola, F. pseudovina*) as matrix species. Altogether 45 native plant species were introduced in September 2014 as seed in different amount. Seeds originated from different sources including cultivated and wild forms (details in *Table 1*). Here we report on the seedings in two restoration parcels: NW1 seeded by purchased cultivated species and S seeded by collected species. The collected seeds included the self-collected and purchased collected seeds in this study. For the map of restoration area see *Figure 1.*

Studied species

Measurements of all seeded species were not feasible because of the limited amount of seed lots. Therefore, seed mass and germination of twelve native species were investigated in the present study under laboratory conditions and compared with establishment in the field. Principle for species selection was that the sample should represent all three seed origin types (seeds purchased from cultivators, self-collected and purchased collected seeds), grasses and forbs. For further details on the species, please see *Table 1.*

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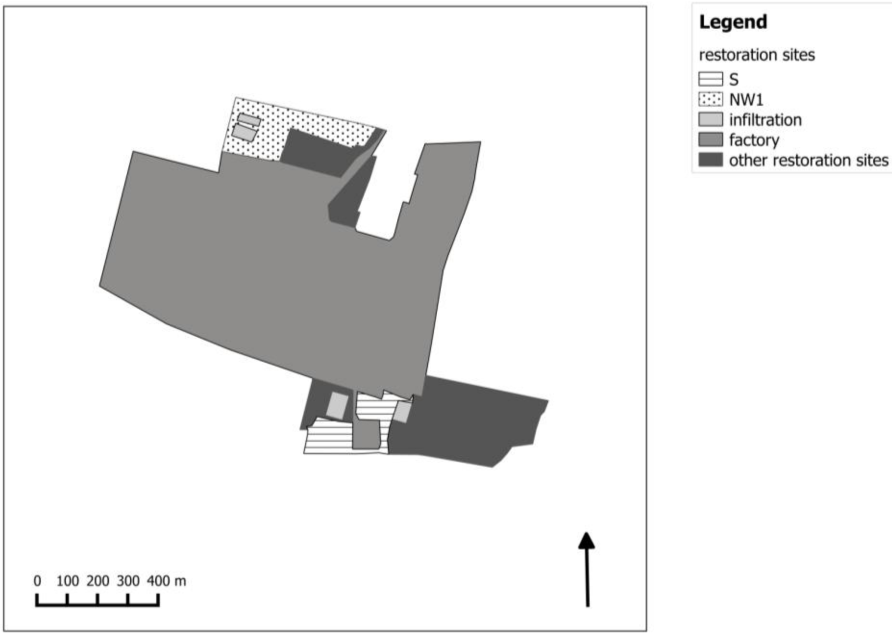

Figure 1. *Map of restoration parcels and treatments within the factory. Establishment of species was estimated in two restoration parcels (named by NW1, S) seeded with 1) cultivated plants (NW1); and 2) collected seeds (S). The collected seeds included the self-collected and purchased collected seeds in this study. In the latter case the amount of hand-collected seeds was not enough to seed the whole parcel, therefore only seven smaller patches were seeded, marked by yellow line. Species list and seeding rates are listed in Table 1.*

Laboratory study

All seeds were harvested in the summer and autumn of 2013 or 2014. Seeds were cleaned and stored at dry conditions (temperature: 25±3 °C, humidity: 38±4%) until the beginning of the germination experiment. All species involved in the study have orthodox seeds, so their long-term viability can be conserved under dry and cool circumstances (Hong and Ellis, 1996). Laboratory tests were carried out on samples from thoroughly mixed, dry seeds. Visibly fertile seed samples of 8 x 100 seeds of each species were counted in January 2015. The dry weight of 100 seeds was measured with 0.0001 g accuracy using an analytical balance. The values of seed mass were calculated as the average of 8 sample results. Laboratory germination of 100 seeds with four replicates per species was tested with and without cold-stratification (Hendry and Grime, 1993; ISTA, 2015). Samples of 100 seeds / Petri-dishes were placed on sterile, wet filter paper. The surface of seeds was not sterilised. Seeds under cold-stratification were placed in refrigerator at 4 °C for a month (Grime et al., 1981; Hendry and Grime, 1993; ISTA, 2015). Seeds without cold-stratification and after cold-stratification were kept under room condition (temperature: 25±3 °C, humidity: 38±4%, light: 3051±939 Lux). The germination experiment started in February 2015 (without cold-stratification), and in March 2015 (with cold-stratification). Seeds were watered by 1-2 ml distilled water every 1-2 days. Germination was defined as emergence of the radicle. The germinated seeds were counted weekly for four months and removed from Petri-dishes.

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Field study

Second year field establishment was estimated in June 2016 in the two restoration parcels (*Figure 1*). Five randomly placed 2 m x 2 m coenological plots per parcel were surveyed by visual estimation of cover (%) by species. In case of species sown into patches, establishment estimation was made by population cover estimation within whole patches. As sowing densities (seed/m^2, *Table 1*) differed between species, according to the availability of seeds, field establishment was calculated as the second-year cover weighted by sowing densities.

Table 1. *List of studied species with origin of seed samples and sowing densities. Seeds were collected in 2013 or 2014 and seeded in September 2014. Origin of seeds: P- seeds purchased from cultivators; CP – purchased collected seeds; CS- self-collected seeds.*

Species	Family names	Origin of seeds	Sowing densities in the field (seed/m2)
Achillea collina Becker ex Rchb.	Asteraceae	CS	2534
Corynephorus canescens (L.) P.Beauv.	Poaceae	CS	123
Festuca pseudovina Hack.	Poaceae	P	7631
Festuca rupicola Heuff.	Poaceae	CP	19496
Festuca vaginata Waldst. et Kit. ex Willd.	Poaceae	CS	28
Galium verum L.	Rubiaceae	P	147
Jasione montana L.	Campanulaceae	CS	323
Poa angustifolia L.	Poaceae	CS	4
Salvia nemorosa L.	Lamiaceae	P	175
Salvia pratensis L.	Lamiaceae	P	63
Securigera varia (L.) Lassen	Fabaceae	P	18
Silene vulgaris (Moench) Garcke	Caryophyllaceae	P	122

Data analysis

Linear model was used to assess the effect of species and cold treatment (explanatory variables) on germination percentage (response variable). Germination percentage was estimated based on four replicates of 100 seeds. This data fulfilled the assumptions of normality. Model residuals showed heterogeneity of variances, therefore generalized least squares (GLS) linear model (Zuur et al., 2009) was used by using the *nlme* package (Pinheiro et al., 2016) with varIdent variance structure that allowed for different residual spread for each level of the species variable. Finally, *contrast* package based on Wald test was used as post hoc test to prove for cold-stratification the significant differences within species (Kuhn et al., 2013).

The impact of seed germination with or without cold-stratification (explanatory variables) on field establishment (response variable) was analysed by two separate generalized linear models (GLMs) with Gaussian distribution and identity link function (Zuur et al., 2009). Average values of germination percentage per species were used. Field establishment was square root transformed before analyses to fulfil the assumptions of normality and homoscedasticity. All analyses were carried out in the R 3.3.1 statistical environment (R Core Team, 2016).

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Results

Seed mass was very different among species. From the studied forbs *Jasione montana* had the lowest (0.0155 g), and *Securigera varia (*4.0269 g) had the highest seed mass. As for the grasses, *Corynephorus canescens* had the smallest (0.0544 g) and *Festuca vaginata* had the largest seeds (0.5546 g) (*Table 2*).

Table 2. *Seed mass, laboratory germination of studied species with or without cold-stratification based on 4x100 seeds and field coverage in 2016 (% of total cover in the sampled plots and patches). Mean seed mass is expressed as thousand seed weight in g (based on 8x100 seeds).*

Species	Seed mass (mean ±SE, g)	Germination with cold-stratification mean±SE (%)	Germination without cold-stratification mean±SE (%)	Field coverage (%)
Achillea collina	0.0727±0.0024	71±2	70±4	1
Corynephorus canescens	0.0544±0.0035	18±2	41±2	0
Festuca pseudovina	0.3931±0.0039	91±1	95±1	47
Festuca rupicola	0.1539±0.0027	40±1	63±4	29
Festuca vaginata	0.5546±0.0102	64±2	78±3	0.02
Galium verum	0.2991±0.0070	6±1	9±2	0
Jasione montana	0.0155±0.0003	79±1	75±8	0.03
Poa angustifolia	0.1939±0.0094	35±3	30±3	0.01
Salvia nemorosa	0.6277±0.0098	1±0	1±1	0.01
Salvia pratensis	1.7581±0.0741	25±5	16±1	0
Securigera varia	4.0269±0.0615	49±3	48±4	0.01
Silene vulgaris	0.5966±0.0200	94±1	89±1	0.51

All species germinated under laboratory conditions, but germination greatly differed (*Table 2*). The highest germination percentages (exceeding 90%) were found for *F. pseudovina* and *Silene vulgaris* both with and without cold-stratification. *Salvia nemorosa* and *Galium verum* germinated in very low numbers (1-9%) under both conditions. Significant interaction was detected between cold stratification and species ($F=13.04$, $df=11$, $p < 0.05$) (*Table 3*).

Table 3. *Effect of cold-stratification and species on germination percentage. Df, F and p values are the result of GLS model. Significant differences are given at p<0.05.*

Explanatory variables	df	F-value	p-value
Cold-stratification	1	7.54	<0.05
Species	11	1869.76	<0.05
Cold-stratification x species	11	13.04	<0.05

The results of post hoc test showed significant response to cold-stratification for five species. Cold-stratification decreased the germination success of four grass species significantly: *Corynephorus canescens, Festuca pseudovina, F. rupicola* and *F. vaginata*. Only one forb species, *Silene vulgaris* germinated better after the cold-stratification (*Table 4*).

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Table 4. *Effect of cold-stratification within species. Df, F and p values are the results of post hoc test, based on Wald test. Significant differences are given at p<0.05, not significant results are marked by n.s.*

Species	t-value	df	p-value
Achillea collina	0.31	72	n.s.
Corynephorus canescens	-8.91	72	<0.05
Festuca pseudovina	-2.39	72	<0.05
Festuca rupicola	-5.45	72	<0.05
Festuca vaginata	-4.06	72	<0.05
Galium verum	-1.30	72	n.s.
Jasione montana	0.48	72	n.s.
Poa angustifolia	1.21	72	n.s.
Salvia nemorosa	-0.31	72	n.s.
Salvia pratensis	1.55	72	n.s.
Securigera varia	0.25	72	n.s.
Silene vulgaris	3.69	72	<0.05

Only nine species of twelve established in the restoration sites by the second year (*Table 2*). The highest plant cover was found for *Festuca pseudovina* (47 %) and *F. rupicola* (29 %). *Corynephorus canescens, Galium verum* and *Salvia pratensis* were not established in the restoration sites. Significant positive relationship was detected between field establishment and both laboratory germination (F=8.52, df=1, p<0.05 and F=7.74, df=1, p<0.05) with and without cold-stratification *(Figure 2*).

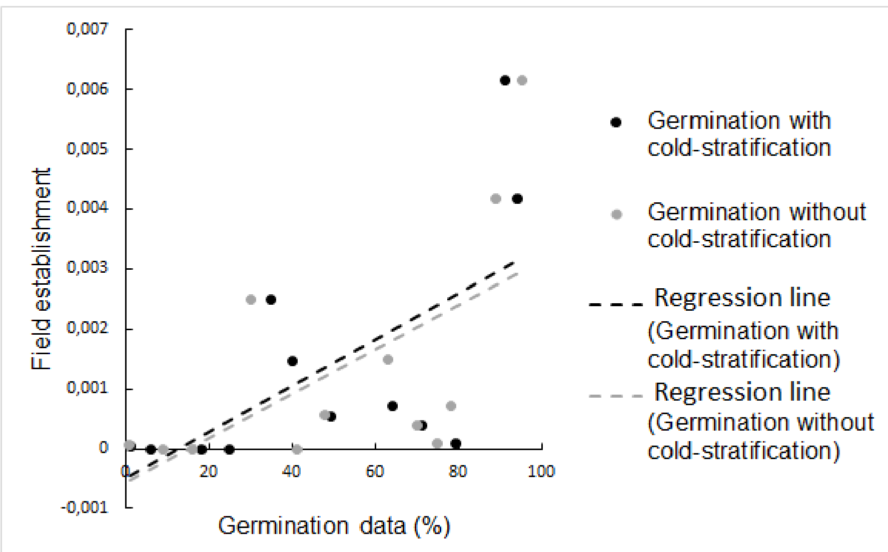

Figure 2. *Relationship between second year field establishment and laboratory germination with / without cold-stratification. Germination was based on 4x100 seeds, field establishment was defined by species cover (%) weighted by seeding rate. Establishment and laboratory germination with cold-stratification (marked by black) and laboratory germination without cold-stratification (marked by gray).*

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Discussion

Importance of cold-stratification for germination

The most broadly used and simplest technique for breaking physiological seed dormancy for native species is pre-chilling, or cold-stratification (Baskin and Baskin, 2004; Russell, 2011; Haslgrübler et al., 2013; Jones and Kaye, 2015). Cold-stratification increased germination potential for only one of twelve studied species in this study. The results are in accordance with literature results concerning grasses that do not require cold-stratification for germination (Partzsch, 2010; Russell, 2011; Hardegree et al., 2013, 2016) since they emerge in autumn (Stampfli and Zeiter, 2008). We found even lower germination percentages with cold-stratification than without cold-stratification for four studied grass species. Forbs respond more variably and may require cold-stratification (Jones and Kaye, 2015; Wang et al., 2016). However, in our study, out of seven forb species we found only one (*Silene vulgaris*) germinating better after cold-stratification. *Silene vulgaris* generally does not require cold-stratification (Liu et al., 2008; Partzsch, 2011), only HUSEED wild database (Peti et al., 2017) supported our contrasting results. The reason might be the different behaviour of different seed samples, as found for *Eriophyllum lanatum* by Russell (2011). In summary, we identified one forb species that need cold-stratification, but most of the studied species did not positively react to this treatment. Other dormancy breaking methods (e.g. mechanical or chemical scarification) should be tested for species with very low germination percentage, like *Galium verum* or *Salvia nemorosa* (Liu et al., 2008; Peti et al., 2017). *G. verum* showed higher germination percentage measured by the Pannon Seed Bank (66% avg. compared to 6-9 % in this study, Peti et al. (2017)), 11 % of *S. nemorosa* seeds germinated compared to 1% in our case, both species after one week of cold treatment. The difference in one order of magnitude can originate from regional differences in population variability. *Salvia* species often require gibberellic acid to break dormancy (Liu et al., 2008).

Germination and second year field establishment

As in previous studies (Clarke and Davidson, 2004; Oliveira et al., 2012; Gallagher and Wagenius, 2016), germination was higher under controlled laboratory conditions than under field conditions. This seems self-explanatory, as field conditions imply further constraints that jeopardize establishment ability. Germinated seeds in the field are challenged by abiotic (drought, inappropriate soil conditions (Leishman, 1999; Clarke and Davidson, 2004; Stampfli and Zeiter, 2008; Carrington, 2014) and biotic (competition with existing species (Leishman, 1999; Clarke and Davidson, 2004; Carrington, 2014; Oliveira et al., 2014), allelopathy or the lack of mycorrhizae (Maltz and Treseder, 2015; Koziol and Bever, 2016) factors to develop further and establish.

Laboratory germination with and without cold-stratification was found to be positively connected with second year field plant establishment in this study. This means that the capacity of seeds to germinate has a long-lasting effect on species establishment. Germination success is dependent among others, on seed age, origin, moisture content, and storage conditions (Mondoni et al., 2013), whereas survival in the field can depend on sowing density, timing and seedling characteristics of co-seeded species (Oliveira et al., 2014). Although seeds were sown in the field prior to winter, when moisture and low temperature conditions are most favourable for germination (Commander et al., 2009),

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only four species were well established (field coverage > 0.5 %), contrary to laboratory results with seven species over 40% of germination success. Studied species, sown in high density were more successful, than others. *Festuca pseudovina* germinated and survived best in the study. This species is a matrix forming grass of closed sand steppe adapted to grazing, and the seeds used for restoration originated from cultivation. Perennial grasses were found to establish better also in other studies compared to forbs (Clarke and Davison, 2004). However, the other tested fescue species (*F. rupicola, F. vaginata*) performed much worse than *F. pseudovina,* and the grey hair-grass (*Corynephorus canescens*) was not even detected in the field. This suggests that other constrains emerged that might be related to e.g. seed origin, collection time, soil requirements or competition. Based on this the choice of dominant grass species for restoration purposes is a major challenge (Gallagher and Wagenius, 2016).

Only three species out of twelve failed to establish or survive till the second year of restoration and five species reached higher cover than 0.5 %. In high diversity mixtures Oliveira and co-workers (2014) found single species being more successful to germinate, establish and grow in the field compared to species mixtures. The performance of target species included in seed mixtures was usually drastically impaired. The unsatisfactory establishment in case of *Galium verum, Salvia nemorosa, S. pratensis* and *Securigera varia,* can be explained in part by the special requirements of seeds for breaking dormancy as explained earlier. On the other hand, traits enabling for fast-growing and competitive advantage of dominant species can supress slow growing species and hinder long-term diversity (García-Palacios et al., 2010). Therefore, we suggest using a matrix of grass species (in this case *Festuca pseudovina* and *F. rupicola*) for the restoration of dry grasslands diversified with a number of non-competitive forbs.

Conclusions and recommendations

Germination performance is usually higher in the laboratory than under field conditions (Oliveira et al., 2012), where environmental conditions (both abiotic and biotic) result in lower germination and establishment. Despite environmental limitation, germination percentage in the laboratory and establishment in the field was found to be correlated in this study. This supports the view that laboratory tests of germination or existing germination databases have the potential to help predict establishment success of selected species.

This study provides results to help unfold the relative importance of germination capabilities of species in determining restoration success, as suggested by Larson et al. (2015), but stepping further by measuring second year establishment in the field. Laboratory germination trials are important to understand and predict the processes in the field (Commander et al., 2009). Germination tests can also help estimate the required seed input. During restoration projects seed introduction of several species occur, but the time and available seed quantities represent constrains for testing the germination capability of all sown species. We suggest carrying out quick laboratory germination tests of dominant species to help identify seeding intensity in restoration programmes. Performance of species with low germination result in the laboratory is very hard to predict in the field. In such cases, we can only remain with the general approach to seed higher amounts in the hope of success, unless further knowledge on dormancy is gained to support decision making in order to cope with future needs (Broadhurst et al., 2016).

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LOW ABUNDANCE OF THE WHISKERED BAT *MYOTIS MYSTACINUS* (KUHL, 1817) IN POLAND – CONSEQUENCE OF COMPETITION WITH PIPISTRELLE BATS?

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Abstract. Interspecific competition is an important factor influencing the distribution of animals, including bats. In this study we tested if the presence of pipistrelles *Pipistrellus* sp. affects the distribution of the whiskered bat *Myotis mystacinus* using information about the species structure of bat communities obtained by capturing with mist nets in whole Poland. Abundance of the whiskered bat is low (on average 3.1%), and it only occurs frequently in the western part of the Carpathian Mountains (24.5%). The negative correlation between the abundance of the whiskered bat and pipistrelles (especially *P. nathusii* and *P. pipistrellus*) in bat communities across Poland seems to support the hypothesis that its occurrence is shaped by competitive interactions.
Keywords: *bat distribution, species range, mountains, sympatric bats, species coexistence*

Introduction

The whiskered bat *Myotis mystacinus* (Kuhl, 1817) has an extensive range in Europe that covers almost the entire continent (Dietz et al., 2009; Benda et al., 2016; Sachanowicz et al., 2016). However, there is no certainty about the eastern boundary of its occurrence due to the difficulty in distinguishing it from the steppe whiskered bat *Myotis aurascens* Kuzyakin, 1935 (Hutson et al., 2008). In Poland, the whiskered bat is widespread throughout the country (Sachanowicz et al., 2006). However its abudance seems to be unequal, with some areas lacking the species (Sachanowicz et al., 2006a) and other regions dominated by them (Piksa, 2011).

Presence of the whiskered bat can be affected by interspecific competition with pipistrelles *Pipistrellus* sp. (Baagøe, 1973). Such processes are key factors shaping the distribution patterns of organisms, as ecologically analogous species sharing a niche cannot occur in the same area (Gause, 1934). This results in the exclusion of one of the competing species (Hardin, 1960), which influences the structure of communities. Interspecific competition is an important factor influencing the distribution of mammals (Ylönen, 1990; Letchter et al., 1994; Eccard and Ylönen, 2003; Bonesi et al., 2006); however it has rarely been considered during the assessment of bat ranges (Arlettaz et al., 2000; Delaval et al., 2005; Santos et al., 2014; Zeus et al., 2017).

In this paper, we attempted to validate the predictions of Baagøe (1973) by comparing the distribution of the whiskered bat with that of pipistrelle species in

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Poland. Our null hypothesis was that the whiskered bat is more abundant in areas where pipistrells are rare or absent.

Material and Methods

To assess the current occurrence of the whiskered bat in Poland we used information on the species structure of bat communities obtained by capturing with mist nets. We analysed both data published since 1997 and our unpublished data from studies conducted since 2000. Initially, we obtained data from 42 locations distributed across the whole of Poland (*Table 1*), and for further analysis we divided these locations into three groups: locations where (1) whiskered bats were not caught, (2) the total contribution of the species accounted for less than 10%, and (3) locations where the whiskered bat accounted for over 10% of all caught individuals. Statistical analyses were performed in R (R Development Core Team, 2009).

Results

The whiskered bat was the most abundant bat species found in southern Poland, and it was most common in the western part of the Carpathian Mountains (*Fig. 1*, *Table 1*) where its average share in bat communities was 24.5% (SE±7.0). In the remaining areas it was rather rare (Welch t-test, t=−3.0315, df=5.059, p<0.05), with its average share in bat communities only 3.1% (SE±0.5).

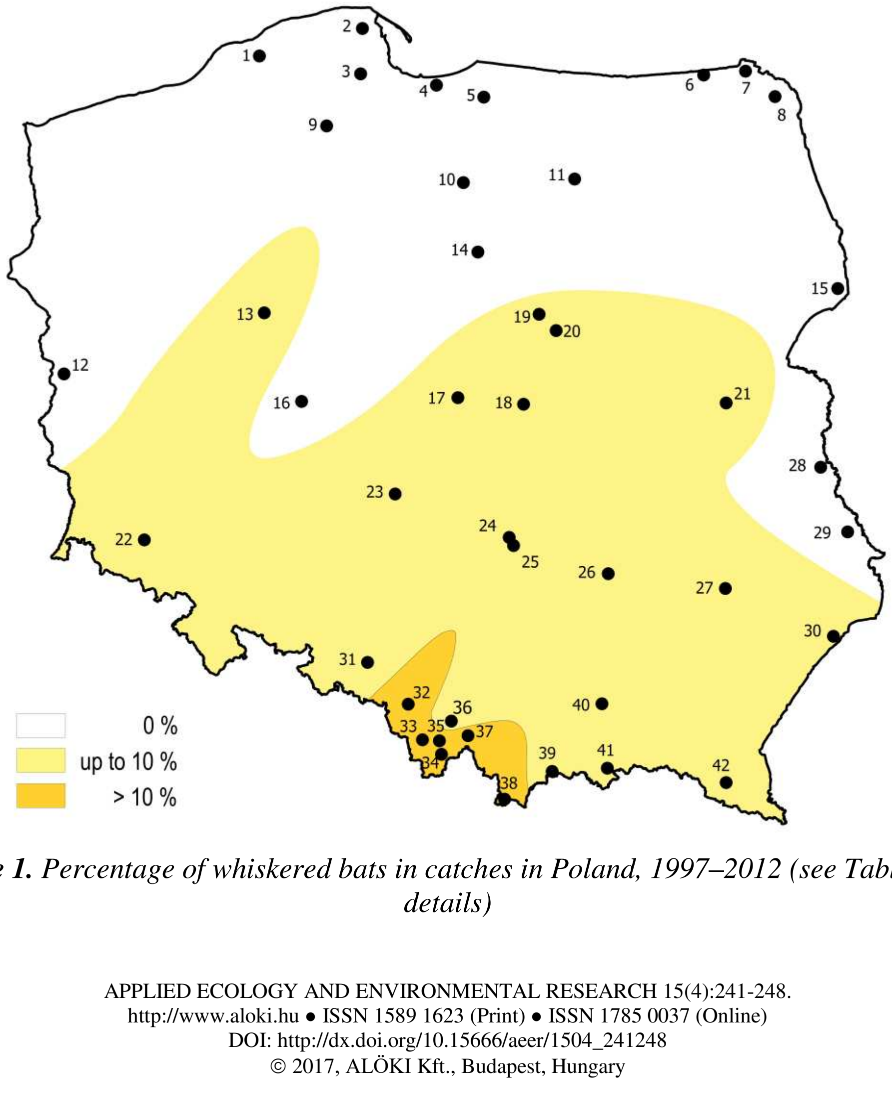

Figure 1. *Percentage of whiskered bats in catches in Poland, 1997–2012 (see Table 1 for details)*

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Table 1. *Percentage of whiskered bat (MYS), Nathusius's pipistrelle (PIN), common pipistrelle sensu stricte (PIP s.s.), soprano pipistrelle (PPY), not distinguished common pipistrelle and soprano pipistrelle (PIP s.l.), all pipistrelles in total (PIPS), and total number of captured bats (N) in various parts of Poland, 1997–2012.*

Area ID	MYS	PIPS	PIN	PIP s.l.	PIP s.s.	PPY	N	source
1	0	13.0	2.0	11.0	8.0	3.0	99	Ciechanowski et al., 2006a
2	0	15.1	10.6	4.5	−	−	132	Ciechanowski, 2002
2	0	16.2	9.3	6.9	−	−	216	Ciechanowski, 2003
3	0	83.9	64.5	19.4	−	−	31	Jarzembowski et al., 1997
4	0	44.5	22.6	−	0.8	21.1	133	Ciechanowski et al., 2008
5	0	15.4	−	−	−	−	87	Authors unpublished data
6	0	3.7	3.7	0	0	0	27	Sachanowicz et al., 2001
7	0	30.3	20.1	2.2	0	8.0	139	Kmiecik et al., 2010
8	0	0	0	0	0	0	146	Postawa and Gas, 2003
9	0	5.0	4.2	0.8	0.8	0	120	Ciechanowski et al., 2006b
10	0	17.6	11.9	0.5	5.2	−	194	Ciechanowski et al., 2002
11	0	6.4	6.4	0	0	0	31	Ciechanowski and Duriasz, 2005
12	0	+	+	−	+	+	?	Cichocki and Łupicki, 2006
13	1.0	3.1	2.4	0.7	0.7	0	289	Łochyński and Grzywiński, 2009
14	0	7.0	6.0	1.0	−	−	84	Kowalski et al., 2001
15	0	13.8	8.5	5.3	−	−	452	Rachwald et al., 2001
16	0	7.4	7.4	0	0	0	27	Łochyński, 2001
17	4.2	5.2	3.1	−	1.0	1.0	96	Hejduk et al., 2008
18	1.7	?	−	−	−	−	231	Hejduk et al., 2004
19	1.5	0.2	−	−	−	−	396	Lesiński et al., 2007
20	1.5	0.3	0.3	0	0	0	400	Lesiński et al. 2006
21	0.8	1.6	0	1.6	−	−	125	Sachanowicz and Krasnodębski, 2003
22	6.8	?	?	?	−	−	44	Dudek et al., 2001
23	3.8	10.0	5	5.0	−	−	131	Ignaczak et al., 2001
24	3.0	?	−	−	−	−	79	Hejduk et al., 2001
25	6.4	16.1	6.4	9.7	−	−	62	Ignaczak, 2003
26	2.3	+	0	0	+	0	2711	Piksa et al,. 2011
27	1.5	0	0	0	0	0	65	Piskorski and Urban, 2003
28	0	5.9	3.4	2.5	1.7	0.8	116	Piskorski, 2008
29	0	5.1	0	5.14	5.14	0	214	Piskorski et al., 2009
30	7.3	4.8	2.4	2.4	0	2.4	82	Piskorski, 2007
31	2.5	16.6	5.1	4.9	1.2	5.4	721	Mysłajek et al., 2002, 2005, 2014
32	18.7	3.3	3.3	0	0	0	91	Authors unpublished data
33	15.9	0	0	0	0	0	1795	Mysłajek et al., 2007 and authors unpublished data
34	21.6	0	0	0	0	0	306	Mysłajek et al., 2010
35	17.9	0.6	0.6	0	0	0	526	Mysłajek et al., 2004 and authors unpublished data
36	8.1	0	0	0	0	0	365	Mysłajek et al., 2013

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37	13.8	0	0	0	0	0	290	Piksa and Gubała, 2011
38	59.3	0	0	0	0	0	3464	Piksa et al., 2011
39	0.8	14.6	0	14.6	–	–	130	Paszkiewicz et al., 1998
40	1.2	0.3	0	0.3	0	0.3	348	Bator et al., 2008
41	2.2	0	0	0	0	0	899	Węgiel et al., 2004
42	3.1	1.2	0	1.2	1.2	0	159	Sachanowicz and Wower, 2013

The abundance of the whiskered bat in Poland was negatively correlated with abudance of pipistrelle species, especially *P. nathusii* and *P. pipistrellus* (*Table 2*).

Table 2. *Spearman's correlation coefficient (Rho) for the percentage of whiskered bats (Myotis mystacinus) in combination with pipistrelles (considered collectively by each species or group of species) in the cohorts of trapped bats in Poland. The table shows the value of the test statistic (S), statistically significant correlation (p-value), and sample size (N).*

Myotis mystacinus in combination with:	Rho	S	p-value	N
Pipistrellus sp.	− 0.589	14519.28	p<0.001	38
P. nathusii	− 0.551	12055.46	p<0.001	36
P. pipistrellus s.l.	− 0.396	9135.34	p<0.05	34
P. pipistrellus s.s.	−0.408	4613.76	p<0.05	27
P. pygmaeus	−0.263	3694.32	ns	26

Disccussion

The observed pattern of whiskered bat abundances in Poland could be explained by their selection for mountainous habitats or intraspecific competition. A factor that may limit the occurrence of the whiskered bat in Poland is the wintering ecology of the species. Whiskered bats hibernate in a fairly broad spectrum of caves; however, they prefers caves located between 1,000 and 1,930 m above sea level (Piksa et al., 2013). Thus, the main wintering grounds of this species can be found at higher altitudes on mountains, where in a study by Piksa et al. (2013), bats from *M. mystacinus* complex group (species are usually impossible to distinguish during winter counts, although the community is probably dominated by the whiskered bat) accounted for 88.5%. The whiskered bat is a species that does not migrate and only undertakes relatively short flights up to a distance of 165 km (Hutterer et al., 2005). Thus, limited abilities for long-distance migration may also limit the distribution and abundance of the species to around the most suitable hibernaculas.

Nethertheless, the presence of mountains alone does not seem to be the most important factor shaping their distribution. Reproducing females were recorded in the lowlands, and the species roosts mainly in anthropogenic shelters and utilizes feeding grounds located primarily in woodlands scattered across agricultural lands (Buckley et al., 2012; Kurek, 2014), which are common in the lowlands. Furthermore, the wide range of the whiskered bat in Europe, which reaches the northern edges of the continent (Hutson et al., 2008), shows that this is a not thermophilic species restricted to the southern and central, i.e. warmer, parts of the continent.

Antagonistic interactions between species most likely explain the observed pattern of whiskered bat distribution in Poland. Gerell (1987), by analysing the density of

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distribution of Brandt's and whiskered bats in northern Europe, suggested that the distribution of these two species is the result of competitive interactions with the common pipistrelle *Pipistrellus pipistrellus* (Schreber, 1774). Our analyses confirmed the constraining effects of at least two pipistrelle species (Nathusius's and common pipistrelle) on whiskered bats. In areas where whiskered bats were captured in large numbers, pipistrelles were not observed at all or were scarce. In addition, pipistrelles were found in these regions beyond the reproductive period (Piksa et al., 2011; Kurek, 2014), which suggests that they were migrating individuals. In other areas, pipistrelle bats are a stable part of the bat fauna (Sachanowicz et al., 2006). Both the whiskered bat and pipistrelles show a preference for similar foraging areas and shelters of anthropogenic origin (Dietz et al., 2009; Buckley et al., 2012; Kurek, 2014), and competition for such resources could be a major factor for the push-out of a significant part of the whiskered bat population to more hostile mountainous areas.

We are aware that the analysis of bat communities based solely on the results of mist-netting could be biased due to a number of factors, including the size and placement of nets, and the frequency at which investigators check the nets (Kunz et al., 2009). Nonetheless, we believe that the potential bias was minimized by using data from numerous studies conducted across the whole country, which includes the repeated sampling of targeted regions, utilization of a variety of netting sites, and use of variable net-sets as suggested by Larsen et al. (2007). Another source of bias may be the use of data obtained during swarming. Places where this phenomenon occurs are called 'hot spots', and during mist-netting researchers capture bats that are not necessarily resident in that given area (Fenton, 1969; Kerth et al., 2003; Bogdanowicz et al., 2012). However, taking into account only catches made in potential feeding grounds, the share of whiskered bats in the southern regions of the country is still estimated to be over 10% (*Table 1*), which supports our findings that the western Carpathians are the main area in Poland where whiskered bats occur.

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EFFICIENCY OF HERBICIDES DOSE IN MIXTURE WITH CYTOGATE FOR WEED CONTROL IN ALFALFA (*MEDICAGO SATIVA* L.)

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Abstract. This study aimed to evaluate the impact of surfactant on herbicide efficiency in *Medicago sativa* L. fields. To this end, a randomized complete block design with 10 treatments and 4 replications was used in three and four-year alfalfa fields, infested with different weeds. The treatments included the standard dose and a 20% reduced dose, a combined treatment and a control sample (weeding and no weed control). Combination of the treatments was applied along with cytogate, and included mixtures of imazethapyr and bentazon, bentazon and pyridate, and imazethapyr and pyridate, with 50% reduced dose of each herbicide. Generally, herbicides applied along with the cytogate surfactant were more efficient for weed control, as well as for an increase of the product yields, as imazethapyr herbicide along with cytogate surfactant managed to control weeds for a rate of 87.43% and increase the product yields to 93.54%. Results showed that combination of herbicides can also be useful in controlling weeds in alfalfa fields and increasing product yields. Results also revealed that combination of bentazon and imazethapyr increased product yields to 94.47% and controlled weeds for a rate of 90.19%. In addition, this method can be proposed as a means to prevent or delay probable resistance to herbicides.
Keywords: *imazethapyr, bentazon, pyridate, reduced dose, surfactant*

Introduction

Alfalfa as a forage crop constitutes the largest area of planting in the world (Lanini et al., 1991; 1999) as Iran (Raoofi and Giti, 2015), and it plays an important role in feeding livestock due to its high protein content (Khanjani and SoleimaniPari, 2005). More than 25% of the dry weight of alfalfa consists of fiber (Meighan et al., 2011).

Unfortunately, particular attention has not been paid to the production of this plant in Iran (Raoofi and Giti, 2015; Raoofi et al., 2013c). One of the challenges of alfalfa production is the presence of weeds (Meighani et al., 2011; Raoofi et al., 2013b). Alfalfa are particularly susceptible to weed competition because they are not vigorous competitors and weeds emerging shortly after seeding can reduce alfalfa success (Zimdahl, 2004).

Weed interference can suppress alfalfa yield (Moyer, 1985; Wilson, 1981) and impact stand densities (Becker et al., 1998). Weeds also alter the composition of the forage, increasing drying time (Doll, 1984) and reducing palatability of the alfalfa (Marten et al., 1987). Generally, weeds will cause severe competition with the crop. In addition to competition with the alfalfa, weed cause a reduction in the quality and quantity of alfalfa and decrease the price by 33% to 60% (Khanjani and SoleimaniPari, 2005; Khanjani, 2000). Similar reduction of alfalfa density due to weeds has been shown by Wilson and Burgener (2009) and Bradley et al. (2010) who reported a 20–

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30% reduced alfalfa density through weed interference. Temme et al. (1979) found that weeds primarily were responsible for decreasing the quality of alfalfa. Frequently, weeds are comparable to alfalfa in quality; however, nutritive quality rapidly declines in weeds as they mature (Doll, 1986). Alfalfa seedlings are particularly susceptible to weed competition because they are not vigorous competitors and weeds emerging shortly after seeding can reduce alfalfa success (Fischer et al., 1988; Zimdahl, 2004). As with any crop, weed competition can reduce yields. Higher alfalfa seeding rate often resulted in greater alfalfa and total forage yield, decreased weed biomass, and increased alfalfa density (Calvin et al., 2011). Weeds interfere with alfalfa during establishment, reducing dry matter yields and plant persistence by competing for light, water, and nutrients (Fischer et al., 1988; Wolfe and Southwood, 1980; Raoofi et al., 2013d). The most damage by weeds in an alfalfa field occurs in the first harvest (Zand et al., 2010; Giti et al., 2013b), however, weeds in many areas, including Hamadan, damage all harvests (Raoofi et al., 2014b). Weed control is often accomplished by using herbicides in alfalfa fields (Myhre et al., 1998). Six herbicides are recommended for using in alfalfa (Zand et al., 2007). Effective use of herbicides is related to several factors, such as time of application, dosage, environmental conditions, method of use, desired plant, frequency of use, etc. Among the techniques to improve the performance of herbicides, use of mixed herbicides, use of integrated methods, none continuous use of one or more herbicides with the same mode of action, use of herbicides with the standard dosage at the right time, and use of additives to improve the performance of herbicides could be named.

Unfortunately, over the past few years, there have been no considerable researches on additives and the context of their use has not been provided practically by farmers. Consequently, only three additives have been recorded by the Pesticides Supervision Board in Iran.

Several herbicides are commonly used in alfalfa. Broadleaf weeds, such as thistles and Brassicaceae, are controlled with 2,4-DB. Flumetsulam and chlorimuron have also been recommended for controlling burning nettle, swinecress, and common chickweed, which are not controlled by 2,4-DB (Lopez and Romera, 1993; Tonks et al., 1991). Imazethapyr is registered for use in alfalfa and non-grazed Conservation Reserve Program lands containing legumes and forage grasses (Anonymous, 2006b). Imazethapyr reduced growth of orchardgrass when applied at the seedling stage and at first cutting (Curran et al., 1999). Paraquat applied during crop dormancy controls many emerged winter annual weeds in alfalfa (Wilson, 1997). These herbicides are safe for alfalfa, but the literature lacks published information on their safety for Dactylis glomerata. In pure orchardgrass (*Dactylis glomerata*), selective herbicides including 2, 4-D, clopyralid, and metsulfuron effectively control broadleaf weeds, but these herbicides cause unacceptable injury to alfalfa (Bradley et al., 2004).

Integrated weed management (IWM) is a combination of effective control methods, which reduces the weed interference to below the economic threshold (Thill et al., 1991). IWM often requires a minimum amount of herbicides (Zand et al., 2009). Evaluation of the impacts of herbicides on the environment is a necessity due to their harmful effects (Campagna, 1995). Unfortunately, the scientific application of herbicides, along with surfactants and their safety has not been studied enough to reduce the use of herbicides. The role of surfactants application were investigated by various researchers (Asmus et al., 2016; Chen et al., 2015). The interaction between the hydrophilic and lipophilic segments of adjuvants for the purpose of increasing the

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efficiency of herbicides has attracted the interest of the relevant researchers (Hall et al., 1999) and remarkable successes have been achieved in application of adjuvants for controlling pests in numerous experiments since 2002 (Zand et al., 2012). This study investigated the use of herbicides with a reduced dosage, along with a cytogate surfactant, and a combination of two herbicides with a 50% reduction of dosage for each herbicide, in addition to the evaluation of weed control and forage production.

Materials and Methods

This experiment was conducted using a randomized complete block design in three and four-year alfalfa fields to investigate the application of chemical treatments with the standard and/or reduced doses (with and without the use of a cytogate surfactant) The study was conducted during two growing seasons of the spring and summer of 2012 and 2013, respectively. Farm under investigation, located at Km 7 Hamadan-Tehran road at latitude of 34° 51' N and longitude 48° 32' E.

Each plot consisted of ten rows with a distance of 25 cm and a length of 4 meters. The distance between the two plots was 60 cm and the distance between the two blocks was 130 cm. For proper evaluation, before performing treatments and concurrent with identifying plots and blocks, three fixed quadrants were installed, each with an area of one square meter per plot.

The design included the treatments with standard and reduced doses (20%), combination treatments and a control plot (complete weeding and no weeding). The treatments were applied in two levels: the standard treatment and reduced doses with the use of a cytogate surfactant. The combination treatments consisted of a mixture of the herbicide treatment with a reduced dose of each herbicide (50%).

The treatment included the use of Pursuit, Basagran and Lentagran herbicides as follows: Pursuit herbicide (0.4 l/hectare) along with cytogate surfactant (0.32 l/hectare), Basagran herbicide (3 l/hectare) along with cytogate surfactant (2.4 l/hectare) and Lentagran herbicide (3 l/hectare) along with cytogate surfactant (2.4 l/hectare). The combination treatments were prepared as a mixture of Pursuit and Basagran herbicides, Basagran and Lentagran herbicides, and Pursuit and Lentagran herbicides, in which a 50% reduced dose of each herbicide was used. In both years of the study, after a 50% flowering in each harvest, sampling took place to determine the weight of the alfalfa, as well as density and dry weight of weeds in a 50 × 50 m quadrat. The dry weight of alfalfa and weeds was determined after placing the alfalfa and weed samples in the oven at 70°C for 48 hours. Statistical analysis software (SAS) v. 9.1 was used for analysis all data, and the obtained averages were compared by means of the LSD least significant difference test.

Results

In the study of two cuttings of established alfalfa, the following weeds were observed and identified, and their distribution levels and importance are as follows, corresponding with the findings of Khanjani and SoleimaniPari (2005) and Raoofi and Giti (2015) (*Table 1*). Environmental condition of the test site are expressed in *Table 2*, and chemical analysis of farm's soil under investigation in the *Table 3*.

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***Table 1.** Alfalfa weeds with their distribution levels and importance*

Scientific name	Family	English common name	Importance value
Carthamus spp.	Astraceae	Safflower	*
Centaurea spp.	Astraceae	Cornflower	*
Ceratocephalus falcatus	Ranunculaceae	_	*
Convolvalus arvensis L.	Convolvulaceae	field bindweed	*
Cuscuta spp.	cuscutacae	Small seed dodder	****
Cynodon doctylon	Poaceae	Bermuda grass	**
Descarainia sophia L.	Cruciferae	Hedge mustard	****
Euphorbia spp.	Euphorbiaceae	Sun spurge	*
Hordeum bulbosum L.	Poaceae	_	*
Hordeum murinam L.	Poaceae	Mouse barley	*
Lactuca spp.	Astraceae	Prickleylettuce	*
Rumex crispus L.	Rosaceae	_	****
Salvia nemorosa	Lamiaceae	Violet sage	**
Sismbrium irio L.	Cruciferae	London rocket	****
Sorghum halepense L.	Poaceae	Johnson grass	*
Taraxacum officinale	Astraceae	dandelion	**
Tragopogon spp.	Astraceae	goatsbeard	**
Vaccaria pyramidata Medic.	Caryophyllaceae	vaccaria	*

***Table 2.** Environmental conditions of the test site*

Operative Measurement	Appraisal
Absolute maximum air temperature	36.8 °C
Absolute minimum air temperature	-29.6 °C
Average air temperature	9.6 °C
The hottest months of the year	July and August
Average air temperature of hottest months of the year	35°C
The Coldest months of the year	December and juneary
Average air temperature of coldest months of the year	-25.4 °C
The annual amount of precipitation	300 ml.
The number of frost days	143 Days
Wind direction	multifarious

***Table 3.** Chemical analysis of farm's soil*

Soil contexture	Sand (%)	Silt (%)	Clay (%)	Potassium absorbable (ppm)	Phosphor absorbable (ppm)	Organic carbon (%)	Epicene material (%)	PH	EC (ds/m)	Depth of sampling (cm)
Silty loamy	33	40	27	332.9	26.4	0.58	9.5	7.6	0.298	30

In this two-year field study, the following weeds were identified in high-density fractions: normal flixweed (*Descarainia sophia* L.), sorrel (*Rumex crispus* L.), bitter flixweed (*Sisimbrium irio* L.), dandelion (*Taraxacum officinalis*) and salsify (*Tragopogon* spp.). These weeds function as the main weeds in alfalfa fields. The effect of the treatments on weed density in all the four of the alfalfa harvests was significant at

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a level of 1% (*Table 4*). The comparison of the average of the treatments with the density of five identified weeds in the first harvest (*Table 5*) showed that the reduced treatment with cytogate and also, the integrated Pursuit and Basagran treatment had the best weed control on bitter flixweed. These results were also consistent with the results of Raoofi and Giti (2015). Normal flixweed was significantly controlled by the use of Pursuit treatments in standard doses, reduced dose of Pursuit along with cytogate, reduced dose of Basagran along with cytogate and combination of Basagran and Pursuit with a 50% reduced dose. It was found that the cytogate consumption as a good surfactant could increase the efficiency of herbicides and decrease their consumption down to 20%. The use of two herbicides, in combination with cytogate, delays the resistance to herbicides in weeds, and therefore, it is highly recommended, since one most important issue regarding herbicide use is cross and multiple resistance (Zand and Baghestani, 2002).

In the investigation of sorrel control in the first harvest, the Pursuit treatments with the standard and reduced doses along with cytogate and also, Pursuit combined with Basagran were analyzed and included in the group with the lowest density of sorrel. Also, in the first harvest, salsify was affected by the Pursuit treatment with the standard dose and had the lowest density. The first harvest of dandelion had the greatest impact from the treatment with a reduced dose of Pursuit along with cytogate and treatment with a combination of Pursuit and Basagran. Raoofi et al. (2013a) also stated that Pursuit along with cytogate is one of the best treatments for broadleaf weed control and can significantly reduce dandelion density. Moreover, Raoofi et al. (2014a) stated that surfactant consumption can significantly reduce the amount of pesticides. Researchers including Ozkan et al. (1993) have evaluated the effects of adjuvants on the performance of pesticide sprays. Some studies have shown that a number of polymers enjoy the ability of breaking down after leaving the sprayer pump in the same way that this process takes place in the return action through the lateral route or hydraulic mixers in ordinary sprayers (Zhu et al., 1997). Comparison of the treatment results in the second harvest (*Table 5*) showed that the best option to control bitter flixweed was the same as the option in the first harvest. The only difference was that the reduced dose of Basagran and accompanying cytogate, the Pursuit and accompanying cytogate treatment and a reduced dose of the combined Pursuit-Basagran were all placed in the same statistical group. These results point to the possibility of reducing the dose of herbicide. Gressel and Segel (1990a, 1990b) also emphasized reducing the use of this herbicide to 0.1 in the standard dose along with the cytogate surfactant. The results of the second harvest and control of normal flixweed were fully compatible with the first harvest. Sorrel, in the second harvest, had the largest decline using the Pursuit treatment with standard and reduced doses along with cytogate, and also the reduced dose of Basagran with cytogate and the Pursuit and Basagran combination. Investigation of the salsify weed density in the second harvest revealed that both the reduced doses of Pursuit and Basagran with cytogate surfactant along with the Pursuit-Basagran combination treatment were placed in the same group. The results of the second harvest for dandelion were fully in accordance with the first harvest results. It was found that the weed density in the second harvest had an exponential decline compared to the first harvest, but the presence of weeds was still evident in the second harvest. It should be noted that the decline of normal and bitter flixweed in the second harvest could have been related to the first harvest due to the cutting of their roots.

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Table 4. *Analysis of variance of the density and dry weight of weeds in each forth harvests. ns,* and ** are insignificant and significant at confidence level of 1% and 5%, respectively*

		Mean of squares							
The source of changes	**Degrees of freedom**	**Harvest density (first)**	**Harvest density(second)**	**Harvest density(third)**	**Harvest density(forth)**	**Dry weight (first harvest)**	**Dry weight (second harvest)**	**Dry weight (third harvest)**	**Dry weight (forth harvest)**
Block	3	1.66^{ns}	1.93^{ns}	1.39^{ns}	1.66^{ns}	0.88^{ns}	1.68^{ns}	1.11^{ns}	0.87^{ns}
Treatment	9	109.210**	111.210**	118.210**	108.210**	476.6**	745.7**	848.4**	518.3**
Errors	27	18.8	19.12	12.42	11.44	22.32	21.24	24.02	22.22
CV		4.11	3.97	4.94	3.88	5.96	5.12	4.52	4.77

Table 5. *The average of weeds density in the first and second harvest during two years. In each column, the average that at least one common letter are not significantly different*

Treatment	**Bitter flixweed (plants/m²)**		**Normal flixweed (plants/m²)**		**Sorrel (plants/m²)**		**Salsify (stem/m²)**		**Dandelion (stem/m²)**	
	Harvest 1	**Harvest 2**	**Harvest 1**	**Harvest 2**	**Harvest 1**	**Harvest 2**	**Harvest 1**	**Harvest 2**	**Harvest 1**	**Harvest 2**
Pursuit 0.4 1/hectare	2.5^{e}	2.8^{d}	3.4^{e}	2.9^{e}	2.4^{e}	2.2^{e}	1.4^{f}	3.5^{e}	3.2^{f}	2.8^{f}
Basagran 3 1/hectare	3.6^{d}	2.9^{d}	5.1^{d}	4.4^{d}	3.7^{d}	3.2^{d}	5.9^{d}	5.3^{d}	4.1^{e}	3.6^{e}
Lentagran 3 1/hectare	10.5^{b}	9.5^{b}	13.5^{b}	13.1^{b}	11.8^{b}	10.6^{b}	12.6^{b}	11.5^{b}	9.9^{c}	8.9^{c}
Pursuit 0.32 1/hectare	1.4^{f}	1.1^{e}	3.1^{ef}	2.8^{e}	1.9^{e}	1.8^{e}	3.1^{e}	2.7^{f}	1.9^{g}	1.6^{g}
Basagran 2.4 1/hectare	2.3^{e}	1.9^{e}	3.1^{ef}	2.6^{e}	3.4^{d}	1.9^{e}	3.4^{e}	2.9^{f}	3.2^{f}	2.8^{f}
Lentagran 2.4 1/hectare	7.8^{c}	7.2^{c}	11.8^{c}	10.6^{c}	10.1^{c}	8.6^{c}	9.7^{c}	9.1^{c}	8.8^{d}	7.9^{d}
Pursuit 0.2 1/hectare +Basagran 1.5 1/hectare	1.4^{f}	1.1^{e}	3.2^{ef}	2.8^{e}	2.2^{e}	1.6^{e}	2.9^{e}	2.8^{f}	2.1^{g}	1.7^{g}
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	11.1^{b}	10.8^{b}	14.2^{b}	13.3^{b}	12.1^{b}	10.7^{b}	13.9^{a}	13.2^{a}	11.3^{b}	10.1^{b}
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	11^{b}	9.6^{b}	14.2^{b}	12.9^{b}	12.1^{b}	10.1^{b}	14.2^{a}	11.5^{b}	10.5^{c}	9.4^{c}
Weedy	14.5^{a}	14.1^{a}	17.3^{a}	15.7^{a}	14.8^{a}	13.1^{a}	14.4^{a}	13.5^{a}	13.8^{a}	12.4^{a}

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One of the non-chemical methods of weed control mentioned by Khanjani and SoleimaniPari (2005) is root cutting in the alfalfa field. According to the literature, there is no e investigation on sensitivity, tolerance or resistance of weeds to root cutting, except for grass at different growth stages (McCarthy et al., 2001). Thus, this study should be replicated on other weeds. The harvesting of alfalfa causes root cutting of the flixweed and thus, can be very effective in reducing flixweed density.

Alternation and repetition of eradication techniques, along with actions such as application of herbicides can increase the effectiveness of weed control. Accordingly, Ghorbani et al. (2009) stated that harvesting and eradicating accompanied with chemical pesticides improve the ability to control weeds. The two weed types, bitter and normal flixweed, were obviously reduced in the third harvest compared to the first and second harvest; however, some weeds still remained on the farm.

The results obtained from the third harvest conformed to the two previous harvests and revealed the favorable effect of the Pursuit and accompanying cytogate treatment. In fact, the controlling effect of the Pursuit and accompanying cytogate treatment was observed even after the study period. Comparison of the means of the experimental treatments on normal flixweed in the third harvest showed that the three Lentagran treatments with standard dose, Pursuit and Basagran with reduced dose and accompanying cytogate were placed in the same statistical group. Pursuit treatments with standard dose, Pursuit with reduced dose and accompanying cytogate and Basagran with reduced dose and accompanying cytogate, were the best and most successful treatments for sorrel weed control in the third harvest. The best treatments for salsify weed control in the third harvest were treatments with reduced herbicide dose, so that both Pursuit and Basagran treatments with reduced dose and accompanying cytogate had the greatest impact and were located in the same statistical group. Also, the reduced dose of Pursuit and accompanying cytogate consumption had a highly positive effect on controlling the dandelion weed in the third harvest (*Table 6*). This treatment also acted favorably in controlling dandelion in the first and second harvests. The study results were also in conformity with Beckie and Morrison's (1993) results and revealed that the reduced doses can be used in weed management if another agent compensates for such dose reduction. Application of Cytogate as an adjuvant in the chemical control of weeds on Alfalfa (*Medicago sativa* L.) has been evaluated before, and its synergistic effects have been confirmed (Raoofi and Giti, 2015; Giti et al., 2013b).

The results obtained from comparison of the means of the treatments in the fourth harvest (*Table 6*) showed that the best options for bitter flixweed control were Pursuit and Basagran treatments, both with a reduced dose and accompanying cytogate. Also, Lentagran treatment with the standard dose, as well as Pursuit and Basagran both with a reduced dose and accompanying cytogate were the best options for normal flixweed control. It should be noted that the problem of bitter and normal flixweed in the fourth harvest was almost insignificant and negligible. Existence of so little density of bitter and normal flixweed in the fourth harvest has been proven by Raoofi and Giti (2012), Khanjani and SoleimaniPari (2005) and Giti et al. (2013a). In a study on the density of these two weeds from the first harvest to the fourth, it was observed that, in the fourth harvest, the weed problem

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was nearly resolved and thus, there was little density of the two weeds in the control (no weeding). The density reduction in the two weeds can be related to eradication of these two weeds resulted from each harvest and passage of the growing season for the weeds, as well as the application of the experimental treatments. The Pursuit treatment accompanied with cytogate in the fourth harvest was introduced as the best option to control sorrel and dandelion weeds. Also, consumption of Basagran with a reduced dose and accompanying cytogate surfactant could transcend the other treatments to control the salsify weed in the fourth harvest.

The results obtained from variance analysis related to the dry weight of weeds showed that all the applied experimental treatments were statistically significant at the level of 1% in all the four harvests (*Table 4*). The results of the comparison of the means of dry weight in the first harvest, considering separated species, *Table 7* showed that the best choice for reducing the dry weight of bitter flixweed was the Pursuit treatment with reduced dose along with cytogate, as well asthe combination treatment of Pursuit and Basagran. Also, Pursuit with reduced dose along with cytogate caused the lowest dry weight of normal flixweed in the first harvest. Investigating the dry weight of sorrel in the first harvest, Pursuit and Basagran treatments, both with reduced dose along with cytogate, and also the combination treatment of Pursuit and Basagran were all in the same statistical group, and were the best treatments for reducing the dry weight of this weed. Pursuit treatment with reduced dose along with cytogate and the combination treatment of Pursuit and Basagran in the fourth harvest were in the same statistical group containing the lowest dry weight of salsify. The Pursuit treatment with reduced dose accompanied with cytogate consumption was the best option to reduce the dry weight of dandelion in the first harvest. Raoofi and Giti (2015) have also recommended these treatments for reducing the weed dry weight.

The results obtained from comparison of the means of the dry weight characteristic in the second harvest, considering separated species, (*Table 7*) showed that the best options for reducing the dry weight of bitter flixweed were the Pursuit and Basagran treatments, both with reduced dose and accompanying cytogate. The combined treatment of Pursuit and Basagran was placed in the same statistical group. The obtained results showed that no antagonistic effects were observed as a result of the combination of the two above-mentioned herbicides. The Pursuit treatment with standard dose and reduced dose accompanied with cytogate along with the Basagran treatment with reduced dose accompanied with cytogate caused the lowest dry weight of normal flixweed (*Table 7*). Investigating the dry weight of sorrel in the second harvest, Pursuit and Basagran treatments with standard dose and Pursuit and Basagran treatments with reduced dose and associated cytogate were in the same statistical group with the minimum weight of dry sorrel. The Pursuit and Basagran treatments with standard dose and Pursuit and Basagran treatments with reduced dose and associated cytogate, as well as the combined treatment of Pursuit and Basagran all led to a minimum dry weight of salsify in the second harvest. The Pursuit treatment with reduced dose and associated cytogate, and also the combined Pursuit and Basagran treatment were the best choices for reducing the dry weight of dandelion in the second harvest. Existence of bitter and normal flixweed dry matter in the third harvest was almost insignificant and negligible; however, among the experimental treatments, the

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treatments of Pursuit and Basagran, both with reduced doses and associated cytogate and weed control treatment contained the lowest dry weight of bitter flixweed and thus, were placed in the same statistical group. Lentagran treatment with the standard dose along with the Pursuit and Basagran treatments, both with decreased doses and accompanying cytogate consumption, caused the lowest dry weight of normal flixweed in the second harvest. In the study of sorrel dry weight in the third harvest, the Pursuit and Basagran treatments with the standard dose, as well as the Pursuit and Basagran, both with reduced dose and associated cytogate, were revealed as the best treatments to reduce sorrel. Effective treatments to control salsify in the third harvest were consistent with the effective treatments to control sorrel, with the difference that the incorporated Pursuit and Basagran treatment associated with the above-mentioned treatments was placed in the same statistical group. Basagran, with both standard and reduced dose as well as associated cytogate, along with Pursuit with standard and decreased dose, as well as accompanying cytogate were the best treatments for reducing the dandelion dry weight in the third harvest.

Mamnooie and Shimi (2012) and Meighani et al. (2011) have also shown that the application of the Pursuit herbicide in alfalfa fields caused significant reduction in weed dry weight. Results of a comparison of the weed dry weight characteristic, considering separated species, in the fourth harvest (*Table 8*) revealed a significant decrease in most of the weeds. A further reduction was observed in both the bitter and normal flixweed, and there was almost no interference problem related to the bitter and normal flixweed in the fourth harvest. However, some weeds were still observed in the fourth harvest. Sorrel, salsify, and dandelion weeds continued to exist until the fourth harvest, because their growth season still caused damage until the late summer. The Pursuit and Basagran treatments, both with standard dose, reduced dose and associated cytogate consumption, caused the lowest sorrel dry weight in the fourth harvest. Also, salsify, as the result of the Basagran treatment with reduced dose and accompanying cytogate consumption had the lowest dry weight in the fourth harvest. Effective treatments on dandelion dry weight reduction in the fourth harvest included the Pursuit and Basagran treatments with standard dose, reduced dose and associated cytogate, which were in the same statistical group. The results of density analysis and alfalfa dry weight in all the four harvests showed that, at the level of 1%, application of the treatment had a significant effect on this characteristic (*Table 9*). The comparison of the means of the experimental treatments showed that the treatments exertion were significantly different from each other, so that from the first harvest until the third harvest, after blank treatment (hand weeding), the Pursuit treatment with reduced dose and associated cytogate consumption accounted for the highest density of alfalfa and was considered the best treatment.

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Table 6. *The average of weeds density in the third and fourth harvest during two years. In each column, the average that at least one common letter are not significantly different*

Treatment	Bitter flixweed (plants/m^2)		Normal flixweed (plants/m^2)		Sorrel (plants/m^2)		Salsify (stem/m^2)		Dandelion (stem/m^2)	
	Harvest 3	Harvest 4	Harvest 3	Harvest 4	Harvest 3	Harvest 4	Harvest 3	Harvest 4	Harvest 3	Harvest 4
Pursuit 0.4 1/hectare	0.1^{c}	0.06^{b}	0.05^{e}	0.01^{b}	1.61^{e}	1.18^{d}	2.25^{e}	1.79^{f}	2.11^{e}	1.46^{d}
Basagran 3 1/hectare	0.26^{b}	0.03^{c}	0.02^{c}	0.01^{b}	2.33^{d}	1.62^{d}	3.38^{d}	2.41^{e}	2.71^{e}	1.79^{d}
Lentagran 3 1/hectare	0.33^{d}	0.01^{e}	0^{f}	0^{d}	2.71^{d}	5.19^{b}	8.09^{b}	5.5^{c}	6.61^{c}	4.36^{b}
Pursuit 0.32 1/hectare	0^{e}	0^{f}	0^{f}	0^{d}	1.21^{e}	0.77^{e}	1.87^{f}	1.47^{f}	1.14^{t}	0.78^{e}
Basagran 2.4 1/hectare	0.02^{d}	0^{f}	0^{f}	0^{d}	1.91^{e}	1.09^{d}	1.36^{f}	0.99^{g}	2.17^{e}	1.44^{d}
Lentagran 2.4 1/hectare	0.1^{c}	0.03^{c}	0.1^{d}	0.01^{b}	6.62^{c}	4.57^{c}	6.21^{c}	4.33^{d}	5.69^{d}	3.66^{c}
Pursuit 0.2 1/hectare +Basagran 1.5 1/hectare	0.26^{b}	0.01^{e}	0.1^{d}	0.01^{b}	7.71^{b}	5.4^{b}	8.79^{b}	6.09^{b}	7.1^{b}	4.21^{b}
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	0.29^{b}	0.03^{d}	0.6^{b}	0.01^{b}	7.92^{b}	5.51^{b}	8.39^{b}	5.52^{c}	6.63^{c}	4.33^{b}
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	0.29^{b}	0.02^{d}	0.1^{d}	0.06^{a}	7.76^{b}	5.49^{b}	8.23^{b}	5.47^{c}	5.4^{d}	4.19^{b}
Weedy	1.4^{a}	0.08^{a}	1.8^{a}	0.06^{a}	11.59^{a}	10.81^{a}	10.97^{a}	11.61^{a}	12.66	11.961^{a}

Table 7. *The average of dry weight of weeds in the first and second harvest during two years. In each column, the average that at least one common letter are not significantly different (LSD α=5%)*

	Dandelion		Salsify		Sorrel		Normal flixweed		Bitter flixweed	
	(stem/m^2)		(stem/m^2)		(plants/m^2)		(plants/m^2)		(plants/m^2)	
Treatment	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1
Pursuit 0.4 1/hectare	$0/55^{cd}$	$0/48^{d}$	$0/63^{d}$	$0/78^{c}$	$0/39^{d}$	$0/78^{c}$	$0/52^{de}$	$0/53^{e}$	$0/64^{c}$	$0/45^{e}$
Basagran 3 1/hectare	$0/56^{cd}$	$0/59^{c}$	$0/99^{cd}$	$0/87^{c}$	$0/84^{cd}$	$0/55^{d}$	$0/87^{d}$	$0/86^{d}$	$0/66^{c}$	$0/63^{d}$
Lentagran 3 1/hectare	$1/7^{b}$	$1/73^{b}$	$2/15^{a}$	$1/96^{b}$	$1/87^{b}$	$1/88^{b}$	$2/14^{b}$	$2/33^{b}$	$1/81^{b}$	$1/92^{b}$
Pursuit 0.32 1/hectare	$0/29^{e}$	$0/24^{e}$	$0/49^{d}$	$0/41^{d}$	$0/4^{d}$	$0/32^{e}$	$0/49^{de}$	$0/36^{f}$	$0/13^{d}$	$0/17^{f}$

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Basagran 2.4 1/hectare	0/51^{b}	0/57^{c}	0/5^{d}	0/79^{c}	0/43^{d}	0/32^{e}	0/33^{e}	0/51^{e}	0/18^{d}	0/43^{e}
Lentagran 2.4 1/hectare	1/66^{b}	1/83^{b}	1/71^{b}	1/89^{b}	1/77^{b}	1/81^{b}	1/77^{c}	1/88^{c}	1/42^{b}	1/46^{bc}
Pursuit 0.2 1/hectare +Basagran 1.5 1/hectare	0/3^{e}	0/54^{c}	0/49^{d}	0/42^{d}	0/29^{e}	0/33^{e}	0/59^{d}	0/56^{e}	0/15^{d}	0/20^{f}
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	1/67^{b}	1/76^{b}	2/2^{a}	2/49^{a}	1/88^{b}	1/83^{b}	2/6^{b}	2/42^{b}	1/87^{b}	1/90^{b}
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	1/68^{b}	1/72^{b}	2/19^{a}	1/99^{b}	1/89^{b}	1/85^{b}	2/35^{b}	2/36^{b}	1/83^{b}	1/83^{b}
Weedy	2/11^{a}	2/17^{a}	2/17^{a}	2/51^{a}	2/33^{a}	2/46^{a}	3/88^{a}	2/85^{a}	2/52^{a}	2/87^{a}

***Table 8.** The average of dry weight of weeds in the third and fourth harvest during two years. In each column, the average that at least one common letter are not significantly different (LSD α=5%)*

Treatment	**Dandelion (stem/m^{2})**		**Salsify (stem/m^{2})**		**Sorrel (plants/m^{2})**		**Normal flixweed (plants/m^{2})**		**Bitter flixweed (plants/m^{2})**	
	Harvest 3	**Harvest 4**	**Harvest 3**	**Harvest 4**	**Harvest 3**	**Harvest 3**	**Harvest 4**	**Harvest 3**	**Harvest 4**	**Harvest 3**
Pursuit 0.4 1/hectare	0/28^{c}	0/41^{c}	0/33^{d}	0/51^{cd}	0/21^{d}	0/32^{cd}	0^{b}	0/01^{c}	0^{b}	0/01^{b}
Basagran 3 1/hectare	0/29^{c}	0/44^{c}	0/39^{d}	0/66^{c}	0/22^{d}	0/55^{c}	0^{b}	0/02^{c}	0^{b}	0/01^{b}
Lentagran 3 1/hectare	0/97^{b}	1/26^{b}	1/17^{b}	1/61^{b}	1/13^{b}	1/51^{b}	0^{b}	0^{d}	0^{b}	0/02^{b}
Pursuit 0.32 1/hectare	0/28^{c}	0/41^{c}	0/32^{d}	0/35^{d}	0/18^{d}	0/32^{cd}	0^{b}	0^{d}	0^{b}	0^{c}
Basagran 2.4 1/hectare	0/29^{c}	0/44^{c}	0/17de	0/31^{d}	0/17^{d}	0/33^{cd}	0^{b}	0^{d}	0^{b}	0^{c}
Lentagran 2.4 1/hectare	0/88^{b}	1/21^{b}	0/19^{c}	1/5^{b}	0/98^{c}	1/5^{b}	0^{b}	0/01^{c}	0^{b}	0/01^{b}
Pursuit 0.2 1/hectare + Basagran 1.5 1/hectare	0/87^{b}	1/22^{b}	1/18^{b}	0/31^{d}	0/99^{c}	1/52^{b}	0^{b}	0/01^{c}	0^{b}	0/03^{b}
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	0/8^{b}	1/24^{b}	1/16^{b}	1/62^{b}	0/98^{c}	1/5^{b}	0^{b}	0/1^{ab}	0^{b}	0/02^{b}
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	0/81^{b}	1/11^{b}	1/17^{b}	1/56^{b}	0/98^{b}	1/51^{b}	0/01^{a}	0/01^{c}	0^{b}	0/03^{b}
Weedy	2/44^{a}	2/66^{a}	2/34^{a}	2/01^{a}	1/9^{a}	2/3^{a}	0/01^{a}	0/2^{a}	0/01^{a}	0/2^{a}

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Table 9. *Analysis of variance of the density and dry weight of alfalfa in each forth harvests. Ns,* and ** are insignificant and significant at confidence level of 1% and 5%, respectively*

				Mean-squares					
CV	**Df**	**Dry weight (forth harvest)**	**Dry weight (third)**	**Dry weight (second)**	**Dry weight (first)**	**Harvest density (forth)**	**Harvest density (third)**	**Harvest density (second**	**Harvest density (first)**
Block	3	144.5 n.s	145.45 n.s	139.51 n.s	144.42 n.s	0.44 n.s	0.42 n.s	0.59 n.s	0.48 n.s
Treatment	10	4221.39**	4251.22**	4211.36**	4206.33**	751.28**	761.51**	736.33**	757.26**
Errors	30	307.12	305.9	306.64	307.68	17.8	19.4	16.6	18.9
CV		5.82	7.22	6.32	7.37	6.14	5.82	6.72	7.31

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The Pursuit herbicide is a chemical treatment of the ALS family, which can be effective in broadleaf weed control. Cytogate can also increase the controlling power of this treatment, as seen in most of the harvests; this treatment could be preferred over other treatments as it appropriately controls weeds. A reduction in the density of weeds provides space for growing of alfalfa and allows it to benefit from the existing resources and conditions. In the fourth harvest, this treatment accompanied with the reduced dose Basagran treatment with associated cytogate consumption was placed in the same statistical group (*Table 10*). In all the four harvests, the Pursuit treatment with reduced dose and associated cytogate was determined to be the desirable treatment for increasing the alfalfa dry weight (*Table 11*). However, a combined treatment of Pursuit and Basagran in the second and third harvests, the Basagran treatment with reduced dose and associated cytogate consumption in the fourth harvest, and the Pursuit treatment with decreased dose and associated cytogate consumption in each harvest were found in the same statistical group and recognized to be among the best treatments for increasing the alfalfa dry weight. The results obtained from the increased pattern of the alfalfa dry weight from the first harvest to the fourth show conformity to the results from the decreased pattern of weeds, so that in the fourth harvest, the density of weed dry weight was observed to be almost the lowest, and the highest alfalfa dry weight was witnessed. In perennial alfalfa farms, in which deep-seated broadleaf weeds exist, Pursuit treatment can be effective in controlling the weeds. In addition, while the decreased dose of the herbicide can be used, such a dose reduction must be compensated for by using a cytogate surfactant so that the amount of poison drift decreases and the control percent increases.

Table 10. *The average of harvest density of alfalfa during two years. In each column, the average that at least one common letter are not significantly different*

Treatment	Harvest density(forth)	Harvest density(third)	Harvest density(second)	Harvest density(first)
	(stem/m^2)			
Pursuit 0.4 1/hectare	147/3^c	145/1^c	144/4^e	131/6^e
Basagran 3 1/hectare	133/3^d	129/7^d	130/1^f	118/96^f
Lentagran 3 1/hectare	97/8^g	98/9^g	98/2^j	89/90^j
Pursuit 0.32 1/hectare	178/5^a	158/8^b	159/4^b	145/15^b
Basagran 2.4 1/hectare	178/8^a	157/9^b	148/3^d	135/39^d
Lentagran 2.4 1/hectare	109/9^f	110/3^f	107/9^i	99/39^i
Weed free	178/9^a	179/1^a	178/8^a	163/77^a
Pursuit 0.2 1/hectare +Basagran 1.5 1/hectare	158/2^b	157/7^b	157/1^c	142/9^c
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	121/7^e	120/0^e	122/3^g	111/19^g
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	121^e	120/7^e	119/7^h	109/66^h
Weedy	96/6^h	99^g	98/7^j	80/07^k

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Table 11. *The average of dry weight of alfalfa during two years. In each column, the average that at least one common letter are not significantly different*

Treatment	Dry weight (forth harvest)	Dry weight (third)	Dry weight (second)	Dry weight (first)
	(g/m^2)			
Pursuit 0.4 1/hectare	138/76^{d}	132/3^{d}	132/7^{c}	117/61^{c}
Basagran 3 1/hectare	120/92^{e}	118/65^{e}	117/26^{d}	105/71^{d}
Lentagran 3 1/hectare	89/75^{h}	89/75^{h}	88/99^{f}	80/72^{f}
Pursuit 0.32 1/hectare	159/72^{ab}	146/35^{b}	145/62^{b}	133/33^{ab}
Basagran 2.4 1/hectare	162/88^{a}	143/24^{bc}	133/11^{c}	117/97^{c}
Lentagran 2.4 1/hectare	99/75^{g}	99/97^{fg}	89/65^{fg}	91/75^{e}
Weed free	165/23^{a}	161/77^{a}	160/73^{a}	137/66^{a}
Pursuit 0.2 1/hectare +Basagran 1.5 1/hectare	149/62^{bc}	142/95^{bc}	145/77^{b}	125/52^{b}
Pursuit 0.2 1/hectare +Lentagran 1.5 1/hectare	110/19^{f}	108/96^{ef}	114/2de	104/77^{d}
Basagran 1.5 1/hectare +Lentagran 1.5 1/hectare	109/92^{f}	108/12^{ef}	113/87de	92/57^{e}
Weedy	88/21^{h}	88/95^{h}	89/55^{fg}	71/88^{g}

Discussion

In general, adjuvants greatly vary with respect to chemical structure and performance, but most of them have a hydrophilic and a lipophilic segment. Herbicides applied along with the cytogate surfactant were more efficient for weed control, as well as for an increase of the product yields, as imazethapyr herbicide along with cytogate surfactant managed to control weeds for a rate of 87.43% and increase the product yields to 93.54%. Results showed that combination of herbicides can also be useful in controlling weeds in alfalfa fields and increasing product yields. Results also revealed that combination of bentazon and imazethapyr increased product yields to 94.47% and controlled weeds for a rate of 90.19%. The equilibrium between the hydrophilicity and lipophilicity of adjuvants is a criterion of the relative distribution of the hydrophilic and hydrophobic components of adjuvants. This important feature has a clear influence on herbicide performance, which was clearly demonstrated in the present research. Certainly, adjuvants are extremely efficient in weed control because they facilitate the spreading of spray particles and absorption of active ingredients and improve herbicide efficiency through reducing surface tension (which was clearly demonstrated in the present study). Since the positive effect of Cytogate in increasing the efficiency of the mentioned herbicides was evaluated and effective results were obtained, it is recommended that this adjuvant should be employed in fields where the spectrum of the mentioned herbicides is used. Although the positive effect of applying Cytogate in improving weed control in fields has been proved, yet it is suggested that other adjuvants be evaluated together with other types of herbicides by researchers so that the synergistic and antagonistic effects of these adjuvants on these herbicides can be determined.

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THE INFLUENCE OF PAST LAND-USE AND ENVIRONMENTAL FACTORS ON GRASSLAND SPECIES DIVERSITY

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Abstract. Mountain grasslands are among the most species-rich plant communities in Europe. This is due to the large variability of abiotic conditions and ways of management. However, a significant change in the rural economy has taken place in recent decades. Some grasslands have become forested, while others have been overgrown as a result of succession. Fodder for domestic animals is produced on grasslands created on former arable land. The aim of this study was to estimate the effect of topographic factors (altitude, slope, exposure) and land use (grassland, arable land) in the mid-nineteenth century and the 1930s on the present biodiversity of mountain grasslands. The study was carried out in the basin of the Łomniczanka and Wierchomlanka Rivers in the Beskid Sądecki (Polish Carpathians). A total of 73 phytosociological relevés in an altitude gradient ranging from 660 to 1,060 m a.s.l were made. Past land use was determined on the basis of archival cadastral and topographic maps. The altitude above sea level was the main factor influencing the botanical composition of vegetation. No difference was observed in species composition depending on past land use. The results show the crucial role of altitude in determining the species composition of mountain meadows.
Keywords: *semi-natural grasslands, grasslands management, species diversity, historical land use*

Introduction

Many habitats, such as semi-natural grasslands, are associated with the centuries-long human activity. The long-term effects of mowing, grazing and fertilization has enabled the emergence of plant communities with numerous species occurring exclusively in these habitats. Mountain grasslands used in a traditional, extensive manner are particularly valuable. These are among the most species-diverse plant communities in the world (Wilson et al., 2012; Chytrý et al., 2015). Grassland communities are also associated with invertebrate species, particularly arthropods (Schaffers et al., 2008). Research also shows that an open landscape is essential for numerous bird species (Siramia et al., 2008; Shi et al., 2014).

The existence of such species-rich communities is the result of the interaction of abiotic habitat factors and extensive use. These communities are found in places with low fertility, which prevents dominance by few highly competitive species, as in fertilized grasslands. On the one hand agricultural production is intensified, while on the other hand marginal areas are no longer being used (Strijker, 2005). Both of these factors lead to a decline in biological diversity and the disappearance of many rare species, which usually have a narrow ecological amplitude (Ellenberg, 1985; Cousins et al., 2015).

In the Polish Carpathian Mountains the problem of abandonment of agricultural land is particularly acute. At the start of the 20th century arable land still dominated the landscape up to an altitude of 1,000 m a.s.l. (Bielecka, 1969) and grasslands occupied

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higher areas or other places that were unsuitable as cropland. In recent decades most former grasslands have become afforested or spontaneous forest succession is taking place on them (Bucała, 2014; Kolecka et al., 2015; Morzyniec et al., 2015). At the same time, arable land has been transformed into grassland, although its total area has not decreased (Twardy, 2008). The grassland communities arising in this manner are usually characterized by lower species diversity (Cousins and Eriksson, 2002).

In mountainous areas the current state of grassland communities is influenced by the high degree of variation in topographic factors such as: altitude a.s.l., exposure, inclination, and soil fertility and moisture. These factors usually act together and it is usually difficult to single out the ones with the greatest effect (Janišová et al., 2010; Klimek et al., 2008). Botanical composition is also linked to the process of immigration of species from other grassland areas and their adaptation to habitat conditions and management. Immigration processes of grassland species, in the case of areas isolated from sources of diaspores, may last a long time. This is most likely why ‘old’ grasslands with a long, uninterrupted history of use are usually richest in species and thus the most ecologically valuable (Reitalu et al., 2010; Pitkänen et al., 2016). The main questions posed in the study were as follows: (i) what is the main factor responsible for the species composition of grassland communities in mountainous areas? (ii) Do grasslands formed on former arable land differ in species composition from those situated in places that were never used to grow crops?

Materials and methods

The study was carried out on clearings located in the Pasmo Jaworzyny mountain range (catchment of the Łomniczanka and Wierchomlanka Rivers), which is part of Beskid Sądecki (Western Carpathians) in the forest zone, in an altitude range of 600–1000 m a.s.l. (*Figure 1*). Bedrock of the Jaworzyna mountain range is composed of flysch sandstones and shales. The soil of all the plots were Haplic Cambisol (Dystric).

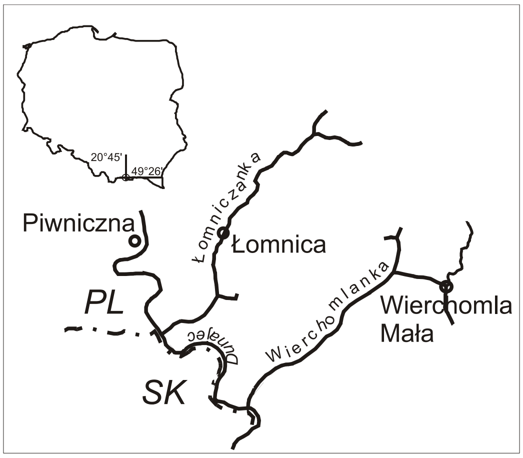

Figure 1. *Localization of research area*

In 2009 73 phytosociological relevés with an area of 100 m^2 were made; a list of plant species was compiled and their coverage were estimated on the five-point scale (Braun-Blanquet, 1964). Species names were taken after Mirek et al. (2002). In each

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place basic topographic parameters were determined: altitude a.s.l., inclination, and exposure. The values for exposure were transformed using trigonometric functions (Roberts, 1986) to create two new variables: northness = cos (exsposure in degrees) and eastness = sin (exsposure in degrees). Cartographic materials were used to determine the type of land use (grassland or arable land) in the mid-nineteenth century, the 1930s and the 1980s. The analysis of past land use was based on Austrian cadastral maps from 1846, a Military Geographical Institute map from 1936 and topographic maps published by the Surveyor General of Poland from 1988. Habitat conditions were evaluated indirectly on the basis of unweighted means of Ellenberg's indicator values (Ellenberg et al., 1992). These were calculated for each relevé for nutrients (N), temperature (T) and soil reaction (R) using JUICE software (Tichý, 2002).

Classification of the phytosociological relevés was based on the similarity of species composition using a modification of TWINSPAN (Roleček et al., 2002). Association of species to vegetation units and statistical significance at $p < 0.05$ (Fisher test) was calculated with JUICE (Tichý, 2002). Syntaxonomic system by Matuszkiewicz (2001) was applied. The ordination analyses were carried out using CANOCO ver. 4.5 software (ter Braak and Smilauer, 2002). In detrended correspondence analysis (DCA) the length of the gradient was 3.6 of the standard deviation and canonical correspondence analysis (CCA) was used to test the effect of topographical factors and use on species composition. Statistical significance was determined by a Monte Carlo permutation test.

Results

Analysis of the phytosociological relevés allowed them to be classified into three main plant communities, forming visible groups with respect to the first DCA axis (*Figure 2A*):

Class: *Vaccinio-Piceetea* Br.-Bl. 1939
Order: *Vaccinio-Piceetalia* Br.-Bl. 1939
Alliance: *Piceion abietis* Pawł. et al.1928

1. Community: *Vaccinium myrtillus*

Class: *Nardo-Callunetea* Prsg 1949
Order: *Nardetalia* Prsg 1949
Alliance: *Eu-Nardion* Br.-Bl. 1926 em. Oberd. 1959

2. Association: *Hieracio-Nardetum* Kornaś 1955 n.n.

Class: *Molinio-Arrhenatheretea* R.Tx. 1937
Order: *Arrhenatheretalia* Pawł. 1928
Alliance: *Arrhenatherion elatioris* (Br.-Bl. 1925) W.Koch 1926

3. Association: *Gladiolo-Agrostietum capillaris* (Br.-Bl. 1931) Pawł. et Wal. 1949

The *Vaccinium myrtillus* community, apart from a large share of this species, was characterized by a substantial share in the area cover of species characteristic of *Hieracio-Nardetum* association: *Nardus stricta*, *Potentilla erecta*, *Deschampsia caespitosa* and herbaceous species: *Hypericum maculatum* and *Luzula luzuloides* (*Table 1*).

This community usually constitutes a stage of overgrowth of unused grasslands and pastures. The number of species in the phytosociological relevé ranged from 7 to 22 (on average 14.1). This community is found in clearings in the upper part of the massif at altitudes from 880 m a.s.l. to at 1060 m a.s.l. (*Table 2*).

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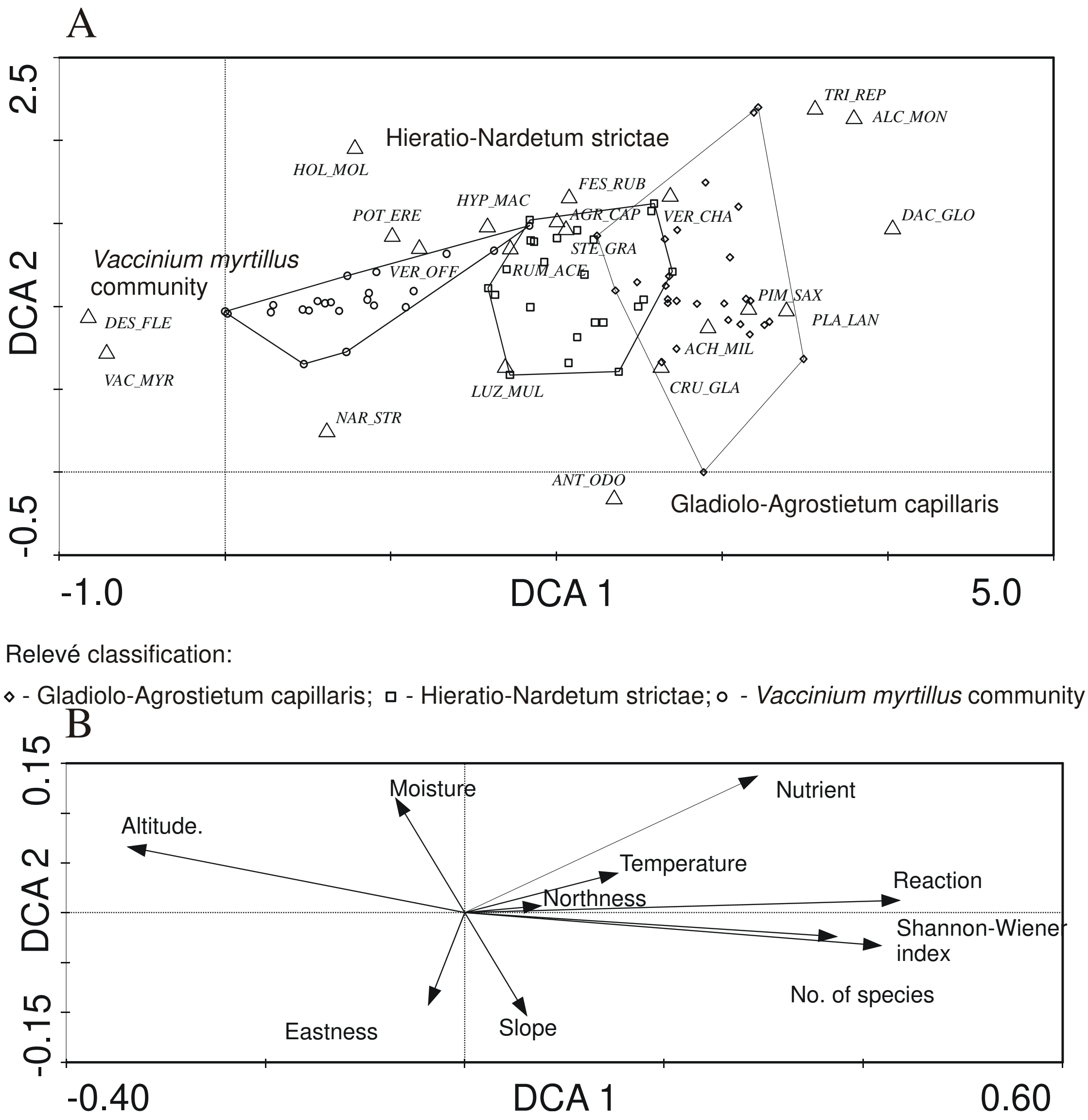

Figure 2. *Ordination diagram (DCA) of the whole data set (eigenvalue of I axis 0.463, II axis 0.127; the length of the main axis gradient is 3.64). A - ordination of phytosociological relevés and species (only species with the highest weight has been presented); B - effect of environmental variables. Species names abbreviation: ACH_MIL - Achillea millefolium, ALC_MON – Alchemilla monticola, ANT_ODO – Antoxanthum odoratum, CRU_GLA – Cruciata glabra, DAC_GLO – Dactylis glomerata, DES_FLE – Deschampsia flexuosa, FES_RUB – Festuca rubra, HOL_MOL – Holcus mollis, HYP_MAC – Hypericum maculatum, LUZ_MUL – Luzula multiflora, NAR_STR – Nardus stricta, PIM_SAX – Pimpinella saxifraga, PLA_LAN – Plantago lanceolata, POT_ERE – Potentilla erecta, RUM_ACE – Rumex acetosa, STE_GRA – Stelaria graminea, TRI_REP – Trifolium repens, VAC_MYR – Vaccinium myrtillus, VER_CHA – Veronica chamaedrys, VER_OFF – Veronica officinalis*

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Table 1. *Species associated with vegetation units (phi indicator for species with statistical significance p < 0.05 Fisher test). Only species with constancy > 60% were shown*

Species	Vegetation unit		
	Vaccinium myrtillus community	*Hieracio-Nardetum* association	*Gladiolo-Agrostietum* association
Deschampsia flexuosa	80.0	---	---
Vaccinium myrtillus	62.4	---	---
Nardus stricta	20.9	12.7	---
Holcus mollis	18.6	13.7	---
Veronica officinalis	0.9	28.7	---
Dianthus deltoides	---	34.6	17.9
Stellaria graminea	---	31.6	20.9
Cruciata glabra	---	30.6	31.0
Campanula patula	---	23.4	26.0
Veronica chamaedrys	---	51.0	40.3
Festuca rubra	---	11.4	14.6
Agrostis capilaris	---	4.9	17.9
Achillea millefolium	---	38.6	41.9
Plantago lanceolata	---	21.9	54.9
Pimpinella saxifraga	---	15.3	55.5
Ranunculus acris	---	4.7	58.6
Alchemilla monticola	---	2.6	56.3
Leucantemum vulgare	---	---	49.8
Phleum pratense	---	---	55.9
Lotus corniculatus	---	---	59.4
Prunella vulgaris	---	---	61.7
Trifolium pratense	---	---	67.7
Trifolium repens	---	---	68.4
Cynosurus cristatus	---	---	86.8

Table 2. *General characteristics of plant communities*

Parameter	Vegetation unit		
	V*accinium myrtillus* community	*Hieracio-Nardetum* association	*Gladiolo-Agrostietum* association
Altitude [m a.s.l.] **	881 - 1064	701 - 969	663 – 897
Slope [°]	0 - 25	0 - 35	5 - 30
Ellenberg's indicator values			
Temperature [T] **	4.7	5.3	5.3
Moisture [F]**	5.2	4.9	5.0
Reaction [R]**	3.5	4.5	5.3
Nutrients [N]**	3.1	3.6	4.1
Total number of species **	48	92	128
Mean number of species in relevé **	14.1	28.8	39.0
Shannon-Wiener diversity index **	1.7	2.6	2.9

** - differences between vegetation units significant at p < 0.01 (Kruskal-Wallis test)

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Patches of multi-species *Hieracio-Nardetum* association are the remains of once dominant in the Beskid Sądecki type of grassland (Pawłowski, 1925). The sward includes a large share of *Nardus stricta* together with other species characteristic of this association, such as *Carex pilulifera*, *Potentilla erecta* and *Veronica officinalis*. Many species can be found here that are also found in *Gladiolo-Agrostietum* association. These include *Achillea millefolium*, *Veronica chamaedrys*, and *Plantago lanceolata*. Often, however, the dominant species are *Festuca rubra* or *Agrostis capillaris* (*Table 1*). This community is found at lower altitudes (700-970 m a.s.l.). The number of species in the phytosociological relevé ranges from 19 to 41 (on average 28.8) (*Table 2*). The community with the greatest species diversity is *Gladiolo-Agrostietum* association. In the patches of this community 128 plant species were recorded, and in individual relevés from 28 to even 55 species (on average 39). Such meadows are usually found in the lower parts of the massif (600–900 m a.s.l.) (*Table 2*). The sward is often dominated by *Agrostis capillaris*, and the presence of species associated with fertile grassland, such as *Dactylis glomerata*, *Trifolium pratense* and *Trifolium repens* is typical. Sporadic use as pasture is indicated by the small but frequent share of *Cynosurus cristatus* (*Table 1*). The indicator values show that the communities do not differ significantly in terms of temperature (T) and moisture (F) requirements. However, substantial differences were noted with respect to soil reaction (R) and nutrients (N), which were highest in the case of the *Gladiolo-Agrostietum* association, and lowest in the V*accinium myrtillus* community (*Table 2*). Analysis of the archival maps indicated that most of the 73 phytosociological relevés were situated in sites that had been used as arable land in the mid-nineteenth century and still in the 1930s. Only in the case of one relevé was it found in a location marked as arable land in the 1980s (*Table 3*).

Table 3. *The number of phytosociological relevés on areas with the same type of land use in the past*

Land use	Period of time		
	mid-nineteenth century	1930s	1980s
Arable land	46	51	1
Grassland	27	22	72

The variation in the species composition of communities present in clearings in the Łomniczanka and Wierchomlanka catchment indicate that altitude was a decisive factor. The grasslands analyzed were separated by short distances, but with considerable variation in altitude, up to 400 m. Increasing altitude above sea level was accompanied by a decrease in species variation, expressed both as number of species and the Shannon-Wiener index, and by an increase in the mean indicator value of the plants for nutrients (N) and reaction (R) (*Figure 2B*). Due to an autocorrelation between the way of management in the 1930s and in the mid-19th century, the analysis took into account only the way of management in the 19th century. The selected variables (altitude, exposure, land use in the past, slope) described 17.58% of the total variance in species composition. Altitude above sea level was responsible for the largest part of it - 9.86% and even for 10.29% after eliminating the influence of the remaining variables (pure effect) (*Table 4*).

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Table 4. *Effect of environmental variables (percentage of explained variation), considered individually (marginal effect) and after deducting the impact of other statistically significant variables (pure effect)*

Environmental variables	Marginal effect	p	Pure effect	p
Altitude	9.86	0.002	10.29	0.002
Northness	2.57	0.014	2.14	0.008
Eastness	1.72	0.028	2.14	0.018
Land use mid-nineteenth century	2.14	0.032	1.72	0.028
Slope	1.29	0.164	-	
Total	17.58		16.29	

Discussion

In contrast with our results, many studies on the biodiversity of semi-natural grassland communities have not shown one main factor influencing species composition (Janišová et al., 2010; Pruchniewicz and Żołnierz, 2014). This may be due to differences in the size of the areas studied. The factors determining species composition in a relatively small area may be different than those acting on a larger scale (Vandvik and Birks, 2002). In the catchment of the Łomniczanka and Wierchomlanka Rivers the variation caused by the altitude above sea level was nearly 10%. This is relatively high, considering that in a study on the effect of factors on the formation of grassland communities in Slovakia the total variance explained by all variables together was only 12% (Klimek et al., 2008). The low percentage of explained variance is typical of data containing many zero values (ter Braak and Verdonschot, 1995), as is usually the case with phytosociological data.

As altitude increased climatic determinants change. The mean air temperature falls by 0.72°C/100 m a.s.l., and the length of the growing period in the conditions of Beskidu Sądecki is reduced by 6.2 day per 100 m a.s.l. (Brzeźniak and Czemerda, 1987), while precipitation increases (Brzeźniak et al., 1984). The indicator values for temperature (T) decreased as altitude increased, but this correlation was weak. This may be due to the relatively low variation in plant species in relation to this factor, as manifested by the large number of species for which no T values are given. Changes in altitude are also accompanied by changes in soil conditions (Manojlović, 2011) differences occur in particular between locations on the sides of mountains and on their hilltops (Maciaszek, 2000). The substantial effect of altitude is linked not only to a change in climate and soil conditions, but also to the possibility and profitability of agricultural use (Tasser and Tappeiner, 2002). Thus the change from arable land to grassland, and then to abandonment of agricultural practices , occurs first in areas that are the most difficult to access, usually at higher altitudes (Twardy, 2008; Tasser and Tappeiner, 2002). Influence of altitude on biodiversity is of common nature and concerned different groups of organisms (Sergio and Pedrini, 2007). Grasslands as semi-natural communities arose in the place of forest and are dependent on mowing or grazing to stop the succession process (Szydłowska, 2010; Hejcman et al., 2013; Tälle et al., 2015). For their existence, apart from suitable soil and climate conditions, diaspores of grassland species must appear. This takes place mainly through anemochory (Tackenberg et al., 2003) and zoochory (Cosyns et al., 2006; Purschke et

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al., 2014), but in either case it takes time (Hutchings and Booth, 1996). This time period may vary, depending mainly on the distance from the sources of the diaspores. In a traditional, alternating system of grassland management in the mountains, when at regular intervals the grassland was ploughed and after a few years grassland use was resumed, meadows and arable fields were present in a spatial mosaic. The flow of diaspores from the meadows to the arable fields was easy and the newly formed meadows rapidly regained a rich species composition (Włodarczyk, 1956). Similar fast process of species immigration to old fields from nearby grasslands was observed in case of xerothermic vegetation (Woch, 2011). In the case of forest clearings this process may last a long time (Winsa et al., 2015). One factor that may influence species composition on former arable land is cultivation procedures, particularly fertilizers that alter the chemical and physical properties of the soil (Chmolowska et al., 2016). For this reason many studies have found that the species composition of grasslands depends on how long the land has been used as grassland (Pitkänen et al., 2016, Purschke et al., 2014). In mountain grasslands in Norway, Austrheim and Olsson (1995) found that the most rapid and greatest changes in species composition took place over a period of up to 23 years from the abandonment of ploughing. Cartographical data indicate that during the period between the mid-nineteenth century and the 1930s there were no fundamental changes in land use in the study area. Arable land stretched as far as to altitudes above 1,000 m. a.s.l. (Kubijowicz, 1927). Fundamental changes in land use took place later. The very small percentage of variance (1.72%) explained by the land management in the past shows that the time elapsed from the transformation of the land into grassland was long enough for typical grassland communities to form. The time needed for the formation of typical grassland communities is highly varied and depends on a number of factors. In the case of xerothermic grasslands the effect of arable land use was demonstrated after more than 100 years (Forey and Dutoit, 2012) while in a study by Chýlová and Münzbergová (2008) the presence of xerothermic communities was noted in places that had been managed in different ways 50 years before. In these studies, however, there was no single factor as strong as altitude above sea level determining the species composition of plant communities. Semi-natural grasslands in a forest zone were created by human activities and their existence depends entirely on their current management (Valkó et al., 2012; Pavlů et al., 2016; Kulik, 2014). Hence, abandonment of farming practices is a more important than the way of land use in the past. *Hieracio-Nardetum* association which dominated in the upper parts of Polish Carpathians in the past (Korzeniak, 2016) has been replaced by V*accinium myrtillus* community as a stage of succession of unmanaged grasslands.

Species-rich *Nardus* grasslands (6230 Natura 2000 code) are currently among the most threatened habitats in Poland and Europe (EIONET) and has been considered as a priority habitat in Natura 2000 network (European Commission). The preservation of such high natural value communities requires their mowing or grazing, which is not possible without subsidies under agrienvironmental programs (Jellinek, 2016).

Conclusions

1. The varied species composition of the grassland communities in the study area was mainly due to the effect of their location at different altitudes above sea level.

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2. The highest species diversity was noted for the *Gladiolo-Agrostietum* association, situated at low altitudes, and the lowest for the *Vaccinium myrtillus* community, located in the higher parts of the massif.

3. As the altitude above sea level increased, the fertility and pH of the soils decreased, expressed as the indicator value of the plant species.

4. The influence of the type of land use in the mid-nineteenth century and the 1930s on the species composition was small. All three types of communities were found in both the fields that had been used as arable land and in those used as grassland in the mid-nineteenth century and the 1930s.

5. None of the plots in the study area (except one) had been ploughed for at least 30 years. This period was sufficient for the formation of grassland communities.

6. The abandonment of plant communities management is one of the most important factors that influenced botanical composition of grasslands.

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MONITORING CHANGES IN URBAN LANDSCAPE PATTERN, USING ALOS AVNIR-2 AND WORLDVIEW-2 IMAGERIES

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Abstract. Recent studies show that urbanization is most affected form of land transformation that intensely influences biological diversity and human life. Quantifying urban landscape pattern is essential for monitoring and assessment of urbanization. The aim of this contribution is monitoring short term changes in urban landscape pattern of Hatay province located in Mediterranean region of Turkey. Hatay is a key region in the ongoing conflict in Syria because of the border and will remain so during the post-conflict transition period. As a result of this situation the urban area has recently undergone extensive land transformations. These land transformations were detected by satellite images of Alos Avnir-2 (2008) and WorldView-2 (2014) using geographic information system (GIS) and remote sensing (RS). Methodology comprised three stages; (1) collecting data and image pre-processing, (2) land cover mapping using object- based image analysis and accuracy assessment (3) landscape change analysis. Landscape change analyses were employed to quantify land cover changes from 2008 to 2014. As a results of the study, forest area decreased from 23,2% to 14,6%. On the other hand urban area increased from 22,5% to 29,9%. This paper demonstrated that urban and forest changed rapidly in this part of the Mediterranean region of Turkey.
Keywords: *Hatay, landscape change analysis, quantify, object-based classification*

Introduction

Monitoring land change science has recently emerged as a fundamental component of global environmental change and sustainability research (Gutman et al., 2004, Rindfuss et al., 2004; Turner et al., 2007; Liu and Yang, 2015; Janisova, 2014). Rapid urbanization and urban sprawl have significant impact on conditions of urban ecosystems. Accurate and updated information on the status and trends of urban ecosystems is needed to develop strategies for sustainable development and to improve the livelihood of cities. The ability to monitor urban land-cover/land-use changes is highly desirable by local communities and by policy decision makers alike. With increased availability and improved quality of multi-spatial and multi-temporal remote sensing data as well as new analytical techniques, it is now possible to monitor urban land cover/ land-use changes and urban sprawl in a timely and cost-effective way (Yang et al., 2011; Güzelmansur and Kılıç, 2013). Since urbanization interferes with many ecological systems and processes, geospatial information on its implications is essential for land use planning and management.

Different classification methodologies and data types have been used for land use/land cover (LU/LC) change detection and database updating. In particular, object-based image analysis (OBIA) techniques enable connecting information from the image with database information (Berberoglu and Curran, 2004; Ming et al., 2015). In OBIA approaches, the pixels are not individually classified but combined into homogenous

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groups (objects) and classified together (Walter, 2004; Mui et al., 2015). Unlike spectral methods employed in pixel based classifications, OBIA are based on segmenting the image into homogeneous pixels (Image objects) and classifying these objects using spectral, spatial, textural, relational and contextual methods. Rather than treating the image as a collection of pixels to be classified on their individual spectral properties, the image pixels can be initially grouped into segments and the object segments can then be classified according to spectral and other criteria, such as shape, size and relationship to neighboring objects. In this way, objects with heterogeneous reflectance values, such as tree canopy, can still be recognized despite their heterogeneity (Zhou and Troy, 2008; Bhaskaran et al., 2010). In comparison with pixel approaches, additional spatial and contextual information can be obtained from objects in OBIA (Blaschke, 2010, Ruiz et al., 2011, Hussain et al., 2013; Wu et al., 2015), which can be easily stored and linked to a database as attributes or features (Gill-Yepes et al., 2016).

Object-based image analysis is quickly gaining acceptance among remote sensors, and has demonstrated great potential for classification and change detection, compared to pixel-based approach (Blanschke, 2010; Myint et al., 2011; Zhou and Troy, 2008; Yu et al., 2016). The advantage of the object-based approach is that it offers new possibilities for image analysis because image objects can be characterised by features of different origin incorporating spectral values, texture, shape, context relationships and thematic or continuous information supplied by ancillary data. Integration of additional knowledge is a valuable means to distinguish ecologically meaningful habitat types that don't have necessarily very distinct spectral features. Moreover integration with existing vector-databases can be achieved during all steps of the classification proces (Bock et al., 2005; Gürkan, 2016)

RS provides a cost-effective alternative to the ground-based survey for land use/landcover mapping and change analysis. (Jensen, 2004; Liu and Yang, 2015; Cserhalmi and Erdős, 2016).

The study employed in hatay province. It is located in the Mediterranean region of Turkey and it is in the border of Syria. So, Hatay is a key region in the ongoing conflict in Syria and will remain so during the post-conflict transition period. Massive population displacements and widespread destruction are linked to the highly strategic nature of the city due to the ethnic and sectarian diversity of the population (Asaad and Shamaly, 2016). Approximatly 500.000 Syrian refugees has been settled to Hatay after conflict acording to local goverments. Some of the refugees settled in the urban area. Therefore, the urban area has recently undergone extensive land transformations. Building development is the most important drive that led to these changes. Hence, the aim of this contribution is to monitor short term changes in urban landscape pattern of Hatay province for 6-year time period between 2008 and 2014. For this purpose, Alos Avnir-2 (2008) and WorldView-2 (2014) images were used.

Urban changes were detected by rectified satellite images of Alos Avnir-2 and Worldwiev-2. The study which performed with the help of RS and GIS mainly consists of three objectives: (1) to land cover mapping using object- based image analysis (2) to make accuracy assesment (3) to quantify land use changes.

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Materials and Methods

Study area

Hatay is a city in the Mediterranean region of the southern part of Turkey, with a population of 500.749 (TÜİK, 2016) *(Fig.1).* The study area is a 8*9 km area including the Hatay city center. It is surrounded by forests of Amanos Mountain in the west, Amik Plain in the north and Habibi Neccar Mountain in the east. Orontes River is the main river crossing the urban area. Climate regime is Mediterranean climate characterized by a mild winter during which about 67 % of the annual precipitation of 1124 mm falls, and a hot dry summer. Average annual temperature reaches a maximum of 44° in the summer and a minimum of -15 C° in the winter, with an average annual temperature of 18 °C. Parent material consists mostly of conglomera, calcaire and alluvial. Mediterranean vegetation consists of evergreen forest of *Pinus brutia*, and shrublands composed of maquis, and garrigues (Guzelmansur and Kılıç, 2013).

Data sets

Alos (Avnir-2): The image collected on 2008 from Advanced Visible and Near Infrared Radiometer type 2 (Avnir-2) of the Alos Avnir-2 were used in this study. The image data has 100 cm spatial resolution and comprise four visible wavebands corresponding to blue (0, 42 to 0, 50 µm), green (0, 52 to 0, 60 µm), red (0, 61 to 0, 69 µm) and near infra-red (0, 76 to 0, 89 µm).

WorldView-2: The image collected on September 2014 from WorldView-2 Ortoready Pansharpened (WV2-NT/ORPNP). The image data has 50 cm spatial resolution and comprise tree wavebands and 16 bites.

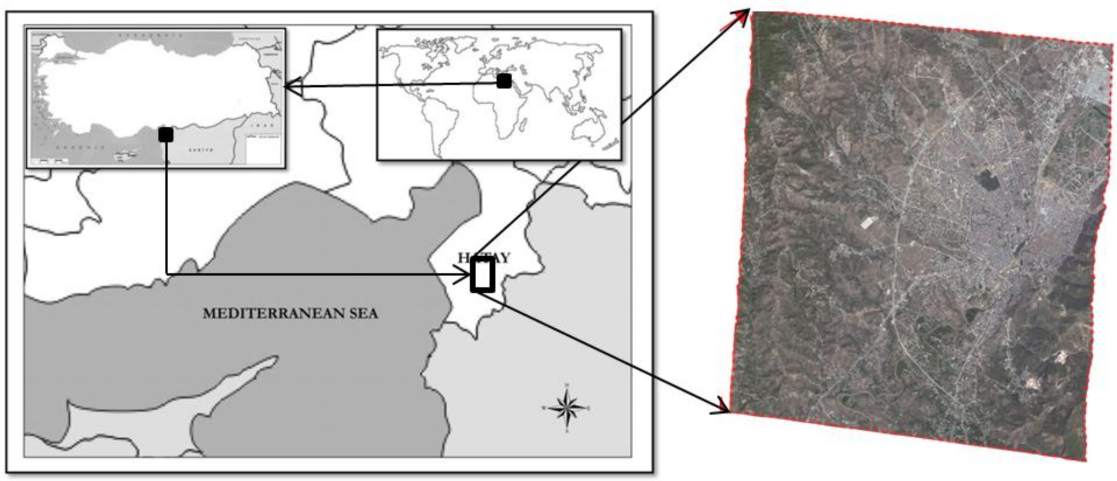

Figure 1. *Site location of study area*

Methodology

The study comprises 3 stages: (1) collecting data and image pre-processing, (2) land cover mapping using object based image analysis and accuracy assesment (3) landscape change analysis.

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Image preprocessing

The image was geometrically corrected and geocoded to the Universal Transverse Mercator (UTM), WGS-84 zone 37 coordinate system using 1/25.000 scale topographic maps. A minimum of 20 regularly distributed ground control points (GCPs) were selected from the image. Resembling was performed using a nearest neighbor algorithm. The transformation had a root mean square (RMS) error of less than <0.5 pixels indicating that the image was accurate within one pixel. A nearest neighbor algorithm takes the value of the pixel in the input image that is closest to computed co-ordinate.

Object-based image classifications

Object-based methodology was adopted for image classification performed for each images captured between 2008 and 2014. Object based image analysis provides rich opportunities for expert (predefined) knowledge integration for the classification process, it also makes the classification more vulnerable to operator-/scene-/ sensor-/target-dependency, which in turn hampers the repeatability and transferability of rules (Witharana and Lynch, 2016). It was preferred in the classification process. Image objects are described and classified by using a wide range of attributes including image features such as spectral variables, shape, texture, size, but also potentially thematic data such as slope, aspect, soil properties provided by digital maps. Image objects may also be classified by reference to expert rules such as rules based on the spatial relationship between objects (contiguity) or the distance between objects (Mathieu et al., 2007). The software used in this research was ArcGIS 10.2. Object-based classification have been recently used in a wide range of application fields (Bock, 2003), landscape pattern analysis (Ivits et al., 2005), fire monitoring (Gitas et al., 2004), land cover/land use mapping in rural (Van der Sande et al., 2003) or urban environments (Meinel et al., 2001) and topographic feature extraction (Repaka et al., 2004; Mathieu et al., 2007; Gürkan, 2016). Seven main categories of land cover types were identified: urban area, agriculture, forest, shrubland, bare soil, water, industry. Additionally, field surveys were carried out to verify these land cover types.

Accuracy assessment

An error matrix, and a Kappa coefficient for each classification were generated. Once a classification exercise has been carried out, there is a need to determine the degree of error in the end-product. These errors could be thought of as being due to incorrect labelling of the pixels. Conversely, the degree of accuracy could be sought. First of all, if a method allowing a 'reject' class has been used then the number of pixels assigned to this class (which is conventionally labelled as “0”) will be an indication of the overall representativeness of the training classes. If large numbers of pixels are labelled as “0” then the representativeness o the training data sets is called into question - do they adequately sample the feature space? The most manly used method of representing the degree of accuracy of a classification is to build confusion matrix (error matrix) (Mather, 1999).

Kappa Statistic is an index that compares the agreement against what might be expected by chance (Eq. 1):

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$$\kappa = \frac{N\sum_{i=1}^{r} x_{ii} - \sum_{i=1}^{r}(x_{i+} * x_{+i})}{N^2 - \sum_{i=1}^{r}(x_{i+} * x_{+i})} \quad \text{(Eq.1)}$$

Kappa coefficient (k) is used to summarise the information provided by the error matrix. The x_{ii} are the diagonal entries of the confusion matrix. The notation x_{i+} and x_{+i} indicates, respectively, are the sum of row I and the sum of column I of the confusion matrix. N is the number of elements in the confusion matrix. Rows total (x_{i+}) for the confusion matrix shown in Table 1 are listed in the column headed (I) and columns totals are given in the last row (Mather, 1999).

Landscape change analysis

Early approaches to measure urban expansion with RS focused on simple band ratios, image thresholding, and image differencing to discern broad-scale changes at the urban–rural fringe (Howarth and Boasson, 1983; Jensen and Toll, 1982; Martin and Howarth, 1989; Shalaby and Tateishi, 2007), while more recent developments accommodate the high spectral variability of urban areas by exploiting spatial or polarimetric dimensions in satellite datasets (Bhaskaran, et al., 2010; Ghimire, et al., 2010; Shaban and Dikshit, 2001; Taubenböck et al., 2012; Shalaby and Tateishi, 2007).

Landscape change analysis was based on image classification. The 2008 and 2014 images classified using object-oriented methodology. Classified images were overlaid to extract landscape change information. Quantitative areal data of the overall land cover changes as well as gains and losses in each category between 2008 and 2014 can be compiled. In order to analyze the rate, and location of land cover changes, a set of 'gains' and 'losses' images for each category was also produced. These 'change' images were overlaid with an image of the city, which was constructed in a vector GIS and it converted into a raster format with the resolution of 10 meters. This GIS overlay intended to find landscape change information within the city.

Results

Land cover mapping

Images of 2008 and 2014 were preprocessed and classified to create land cover maps. The two land-cover maps discriminated seven classes: urban, agriculture, forest, shrub land, bare, water and industry (*Fig. 2*). The urban class included settlement area, cemetery, educational institutions, parks and their roads. Agricultural lands were split in two classes to increase their discrete with other land-cover classes; annual agriculture and horticulture. The forest class included natural forest areas (*Pinus brutia* Ten.) and maquies vegetation. Shrubland is easily sacrificed for different land uses to *Sarcopoterium spinosum* (L.) Spach. The most important water flow of the City is Asi (Orontes) River classified as water. Industry consists from commercial (small industrial site) and the other minor saleable area in the city and it's around.

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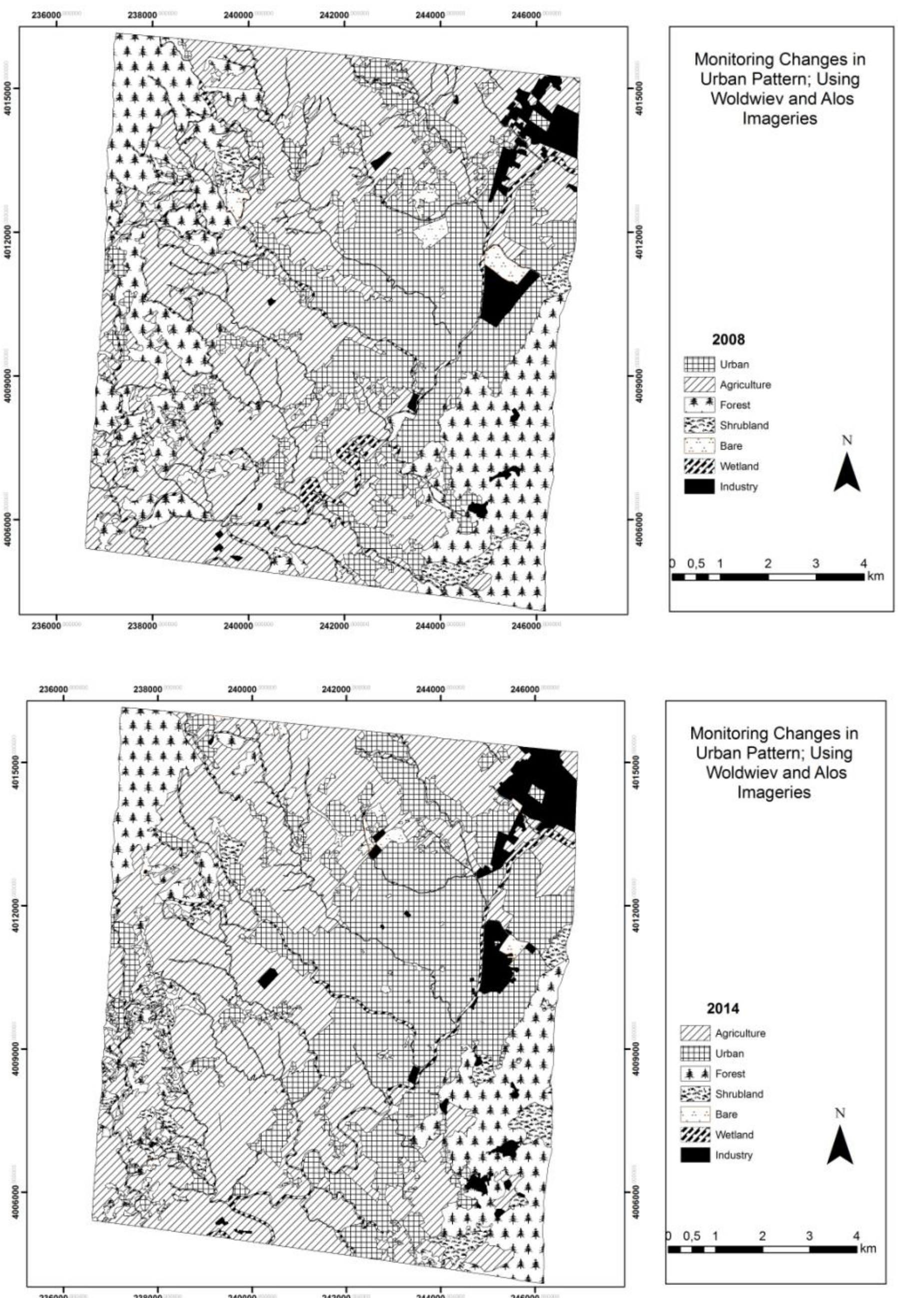

Figure 2. *Land cover in 2008 and 2014*

Validating classification results

Table 1 and *Table 2* shows that indicate the level of consistency between observed land cover for 2008 and 2014. Confusion or error matrix consist for seven classes. The row labels are those given by an operator using ground reference data. The column labels are those generated by the classification procedure. The four right-hand columns are as follows: (I) number of pixels in class from ground reference data; (II) estimated classification accuracy (percent); (III) class i pixels in reference data but not given label by classifier; and (IV) pixels given label I by classifier but not class I in reference data (Mather, 1999). The sum of the diagonal elements of the confusion matrix is for year of

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2008 is 160, and the overall accuracy is therefore (160/179) x 100 = 90,67% (*Table 1*). The sum of the diagonal elements of the confusion matrix for year of 2014 is 185, and the overall accuracy is therefore (185/204) x 100 = 90,68% (*Table 2*).

Overall acuuracies and Kappa values were over %86 in most cases which is acceptable rate for accuracy (Foody, 2010). Water had the highest accuracy values. Forest, urban, bare, industry, agriculture follow respectively. Accuracies for urban areas in 2008 classification are slightly lower than 2014 but still around 90%. Resolution of the image of 2008 lower than 2014, that is why the accuracy is higher than 2008. High classification accuracies showed that, use of enhanced images provided opportunities for mapping this variability effectively. The fact that an object-based image analysis was adopted also helped to produce very accurate classifications.

Table 1. *The sum of the diagonal elements of confusion matrix for 2008*

LU/LC Type	Urban	Agri-culture	Forest	Schru-bland	Bare	Water	Industry	I	II	III	IV
Urban	30	2	1	1	1	0	1	36	88,31	6	3
Agriculture	1	25	1	1	1	0	0	29	86,20	4	5
Forest	0	1	40	0	0	0	0	41	97,56	1	3
Schrubland	0	2	1	25	1	0	0	29	86,20	4	4
Bare	0	0	0	2	15	0	0	17	88,24	2	3
Water	0	0	0	0	0	10	0	10	100,00	0	0
Industry	2	0	0	0	0	0	15	17	88,23	2	1
Total	33	30	43	29	18	10	16	179		19	19

Table 2. *The sum of the diagonal elements of confusion matrix for 2014*

LU/LC Type	Urban	Agri-culture	Forest	Schru-bland	Bare	Water	Industry	I	II	III	IV
Urban	35	3	1	1	1	0	1	43	83,33	7	4
Agriculture	1	30	1	1	1	0	0	34	88,24	4	6
Forest	1	1	40	0	0	0	0	42	95,24	2	3
Schrubland	0	2	1	30	1	0	0	34	88,24	4	5
Bare	0	0	0	3	20	0	1	24	83,33	4	3
Water	0	0	0	0	0	15	0	10	100,00	0	0
Industry	2	0	0	0	0	0	15	17	88,24	2	2
Total	39	36	43	35	23	15	17	204		29	23

Quantifying from to changes

In this study, landscape change analyses technique was applied. It is the most obvious method of change detection, which requires the comparison of independently produced classified images.

Fig. 2 show the result maps of land cover in 2008 and 2014. These resulting maps were investigated and compared. Urbanization increased from 22,5% to 29% which it means approximately 7.000 hectar *(Table 3).* Additionally, agricultural areas icreased from 44,4% to 45,6%. Shrubland and industry also increased from year of 2008 to 2014. On the other hand, forest area decreased from 23,2% to 14,6%. The most prominent change in the study area was replacement of forest by urbanization, agriculture and shrubland. Replacement from forests to urban areas was observed. These changes occured especially around the villages of Ballıöz, Doğanköy, Günyazı and Toygarlı. These areas have been used for recreational activities in the recent years. Additionally,

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the figure show that expansion of agriculture over shrubland, resulted in a decrease in this cover type. Rapid urbanization is evident in the study area.

Table 3. *LU/LC of 2008 and 2014*

LU/LC Type	2008(ha)	2008(%)	2014(ha)	2014(%)
Urban	23.773	**22,5**	30.635	**29,0**
Agriculture	46.941	44,4	48.174	45,6
Forest	24.550	**23,2**	15.423	**14,6**
Schrubland	1.771	1,7	3.113	2,9
Bare	1.047	1,0	989	0,9
Water	4.689	4,4	3.442	3,3
Industry	2.852	2,7	3.846	3,6
Total	105.623	100,0	105.623	100

Analysis results showed that area coverage of various LU/LC types is unbalanced. Agriculture and urban are the most extensive LU/LC types in the study period. Agriculture covered 46941 hectar of land, which made up 44% of the study area in 2008. This reached to 48174 hectar in 2014, corresponding to 46% of total area. Urban cover that occupied 23773 and 30635 hectar of land in 2008 and 2014, respectively, has the second largest extent. Forest areas covered 24550 hectar, in 2008. However, this area decreased because of urban, agriculture and shrubland. It declined from 24550 ha in 2008 to 15423 in 2014. Maquies are trimmed to plantation of olive trees. These cause to replacement from forest to agriculture. Additionally, some recreational activities like picnic, ect. couse the urbanisation of forest area.

Fig. 3 provide information about the LU/LC conversions, which showed that most of the changes took place in urban, agricultural and forest areas. Maquie vegetation and pine forest make up the forest in the study area. As previously mentioned, agriculture-related changes comprised conversions from/to agriculture. Urbanization mostly took place at the expense of agriculture, forest, bare areas, shrubland and industry. The fact that higher rates of urbanization takes place on agriculture areas is an important finding worthy of consideration and it requires attention in development planning for the region. Forest area decreased in the study period of the years from 2008 to 2014.

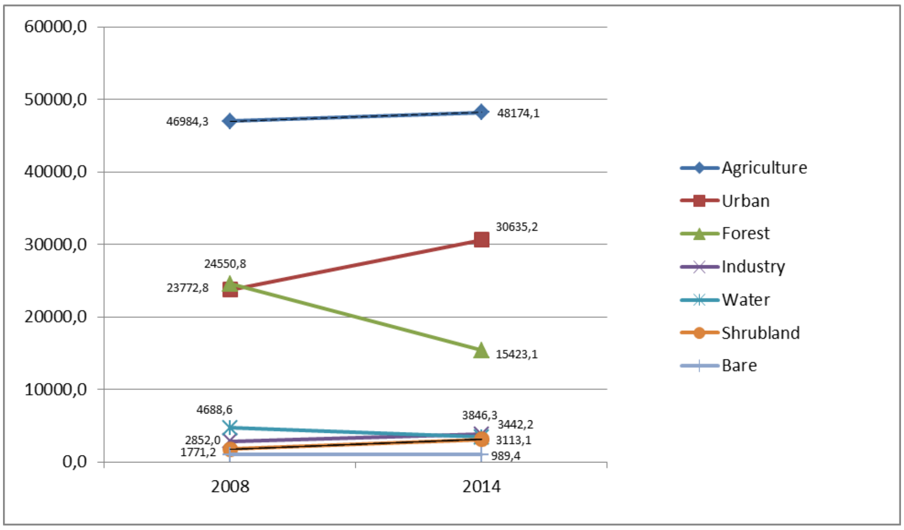

Figure 3. *LU/LC changes from 2008 to 2014*

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Forest areas have been turned into cultivated, urban or shrub areas. Although the same classification method is used in some areas in both images, it is found that forest areas have been transformed into areas used for other purposes as a result of the difference in image resolution of two images. Worldview-2 image resolution was 50 cm while the other was 100 cm. Some shrubland areas around the forest patches classified as forest areas because of resolution of image. This is one reason for the decrasing the forest area from 2008 to 2014. However, urban area has been extensive and some natural areas convert to some cultural land uses such as urbanization and agriculture.

Discussion

Alphan and Çelik (2016), used landscape pattern while Esbah et al. (2010) used landscape stucture metrics. Esbah et al, employed change analyses that highlighted the increase in artificial surfaces and decrease in arable lands and forests in a protected area. Alphan and Çelik (2016), used landscape pattern to monitore changes in coastal zone. They highlited dramatic transformations from agriculture to build-up areas. Similar results were obtained in our study; urban area increased rapidly between 2008 and 2014. However, forest area decreased dramatically between this time period. From environmental planning and management point of view, degredation of forest areas is an important disquiet.

Conclusions

The contribution assessed urban landscape changes using landscape change analysis. The analysis included classifications of two different sensors of Alos Avnır-2 and Worldview-2 which have high spatial resolution. As a consequence of landscape change analysis, there is an increase in urban development and forest related changes occured. This makes it essential to properly analyze changes for establishing appropriate local development strategies and monitoring of development in the urban area. When facing such severe and rapid land-cover changes, one requirement for decision-making is to be able project future changes under certain assumptions. Such projections also contribute to increase awareness of the ecological consequences of growing pressures. However, this study focused on a period of time which is probably too short to understand the land-cover change process in all its complexity. Yet, this period is important because of the ongoing conflict in Syria which led to the settlement of Syrian refegees in Hatay's urban area. Attempts must now be made to increase the period of observation of the past (i.e. before the resettlement) by analyzing longer temporal series of land cover data.

Current remote sensing techniques enable the application of monitoring in temperate regions of the world (Berberoglu et al., 2000; Berberoğlu and Akın, 2009). The contribution presents the results of OBIA classification-based mapping of an area around the city of Hatay in Turkey, using images from two different high resolution sensors of Alos Avnır-2 and Worldview -2. Land cover changes were quantified in terms of percentage of area affected and rates or change.

The results presented in the paper show that OBIA is a valuable method for monitoring changes in urban landscape pattern which are useful for urban planners and may be interpreted to raise awareness for the use of natural resources. OBIA is performing very well when applied to Worldview-2 and Alos Avnir-2 imagery. The

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method provides flexibility in the ability to transfer classification schemes developed recent images where a fair set of ground truth data is available.

High resolution satellite images like WorldView-2 and Alos Avnir-2 images can be used for these kinds of studies. It can save time and effort for fieldwork. But some disadvantage in the study was, if the both images have same spatial resolution the classification results will be more accurate. Additionally there are also some disadvantages such as high price of digital imagery, difficulty in finding qualified staff to work about GIS.

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ASSESSMENT OF SMALLHOLDER FARMER'S VULNERABILITY DUE TO CLIMATE CHANGE IN ARID AREAS OF PAKISTAN

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Abstract. In developing countries, such as Pakistan, the food and financial security of smallholder farmers are increasingly threatened by climate change, contributing to considerable increases in input costs and market price volatility for agricultural commodities. This study aimed to identify and investigate factors contributing to smallholder farmers' vulnerability to climate changes and to develop vulnerability indices in Punjab province, Pakistan. Three different composite indices were used to assess the vulnerability of smallholder farmers to climate changes: Livelihood Vulnerability Index, Livelihood Vulnerability Index-Intergovernmental Penal on Climate Change, and Livelihood Effect Index. The weakness of social networks in Rawalpindi district is majorly important to enable the adoption of appropriate mitigating strategies. For both districts, lack of education had the highest effect on social vulnerability. Adoption of strategies towards mitigation of climate changes seems to be possible by the respondents in Chakwal district, with higher illiteracy rate, higher dependency ratio, and greater financial vulnerability. The natural and financial capital had the highest impact on the livelihoods of smallholder farmers in both districts. Ultimately, this research can provide a scientific basis for the decision-making process and risk analysis of climate change impacts in developing countries, where most of the smallholder farmers, as a major social group worldwide, are located.
Keywords: *environmental change, livelihood, livelihood vulnerability index, sustainable development, Potohar*

Introduction

Agriculture is one of the sectors most sensitive to climate change because any degree of climate change can be associated with severe negative impacts on agricultural production and related processes (Lin et al., 2007). Such impacts are predicted to be particularly harsh in the tropical countries, lowlands, and areas affected by reduced snowfall and melting glaciers. In the tropical countries, any increase in temperature can contribute to a considerable decrease in crop yields due to already reached the threshold of heat and drought tolerance by the crops (Burch et al., 2007). Lowlands can be adversely affected by flooding and soil salinization due to predicted sea level rise and groundwater salinization. The areas impacted by reduced snowfall and melting glaciers can face severe droughts due to a decrease in water availability for meeting crop water requirements (IFAD, 2013).

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For the last two decades, the vulnerability of agricultural production to climate change effects increasingly became the focus of comprehensive research (Tan, 2004). However, despite worldwide recognized the potential of climate change for negative impacts on agriculture, research work on assessment and mitigation of these impacts remains scarce in developing countries, particularly on smallholder farms (Morton, 2007; Ahmed and Schmitz, 2015). It is significantly important because smallholder farmers have been identified as most vulnerable to climate change impacts, both in developed and developing countries (Adger, 2006). Therefore, research on the implications of climate change on agriculture and decrease in farmers' livelihood vulnerability to these impacts in the most exposed regions, as mentioned above, is highly required for developing effective adaptation measures and ensuring sustainable agricultural development. Such research would also provide a scientific basis for the decision-making process and risk analysis of climate change impacts in the investigated locations (Tao et al., 2011).

Smallholder farmers are a distinct social group regarding numbers and food security. They are estimated at 400-450 million of world population and 87% of Asian population (Von Braun and Pandya-Lorch, 2007). They are also assessed as representing the insecure food half of global population (Nagayets, 2005; Sanchez and Swaminathan, 2005). The term ''Smallholder Agriculture'', as used in under-developed countries, commonly defines rural producers who generate income from the family labor farming (Cornish, 1998) and hold less than 2 hectares (ha) of land (Csaki and de Haan, 2003). The food security is increasingly threatened by climate change effects, such as severe pest breakouts, extreme weather conditions, and water scarcity, contributing to considerable increases in input costs and market price volatility for agricultural commodities (O'Brien et al., 2004; Morton, 2007; Makondo et al., 2014) . In this light, and considering agriculture as primary source of their income, smallholder farmers are particularly vulnerable to climate change impacts. Such as the above mentioned ones, mainly due to high risk of yield losses, with further negative impacts not only on their food but also financial security and, ultimately, in their living conditions (Rosch and Hertel, 2010; McDowell and Hess, 2012)

Pakistan is a developing country where agriculture contributes 20.9 % to GDP and 43.5% of the national labor force, but with generally little opportunity for income generation and small market returns in the rural areas (Farooq and Wasti, 2015). The climatic conditions are characterized by arid and hot climate, with the occurrence of droughts and floods negatively impacting on agricultural production (Yu et al., 2013). In this light, and considering the farmers' low level of awareness of climate changes, it can be deduced their high vulnerability to these changes and the need to assess this vulnerability.

In this context, this work aimed to identify and investigate factors contributing to smallholder farmers' vulnerability to climate changes and to develop and adapt vulnerability indices in two districts of arid region in Punjab province, Pakistan.

Methods

Study area

This study combines methods from different schools of climate change assessments to assess livelihood vulnerability in two districts, Chakwal and Rawalpindi, of the arid region in Pakistan. This region has been considered as having the considerable

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agricultural potential to reduce imports of agricultural commodities at the national level (Ijaz and Ahmad, 2006). Fragmentation characterizes the agricultural land, and the region contributes around 10 percent of national aggregated agricultural production (Ashraf et al., 2004). Chakwal district has an estimated population of 1.4 million within an area of 6.524 Km^2 (Rafique, 2015). There are 151989 farms in this district, out of which 65 percent are considered as smallholder farms and occupy 29 percent of the cultivated area (Pakistan-Bureau-of-Statistics, 2010). The climate is arid, characterized by hot and dry summers and cold and dry winters. The average annual rainfall is 880 mm. The average temperature is 8ºC during winter, rising to 42ºC during summer. The landscape is diverse, with plains and hills (Sheikh, 2012a). Rawalpindi district has an estimated population of 4.7 million within an area of 5.285 Km^2 (Rafique, 2015). There are 182186 agricultural farms in the district, out of which 85 percent are smallholder farms occupying 41 percent of the total cultivated area (Pakistan-Bureau-of-Statistics, 2010). The climate varies from district to district, as winter is remarkably cold, and summer is mild in Murree Tehsil, whereas summer is tolerably hot, and winter is mild in Gujar Khan and Rawalpindi tehsils. The average annual rainfall in the district is 1550 mm. The landscape is dominated by plains in Gujjar Khan and Rawalpindi tehsils (Sheikh, 2012b). In both districts, the major known crops are wheat, groundnut, rapeseed, and mustard, with additional vegetables such as onion, potato, tomato and peas.

Data collection and analyses

Household survey, of 120 randomly selected smallholder farms with less than 2 hectares, was used for data collection. Three different composite indices were used to assess the vulnerability of smallholder farmers to climate change: Livelihood Vulnerability Index (LVI), Livelihood Vulnerability Index- Intergovernmental Penal on Climate Change (LVI- IPCC), and Livelihood Effect Index (LEI). The methodology used for calculating the indices was adapted from (Hahn et al., 2009) and (Khajuria and Ravindranath, 2012), so as to include specific climatic and contextual conditions (Parry et al., 2007). In this study the index LEI is constructed on Sustainable Livelihood Framework to understand vulnerability regarding livelihood. The framework for sustainable livelihoods has been presented in *Fig. 1* which shows that processes and structures, livelihood outcome and livelihood strategies of a community are influenced by the vulnerability context and that is determining factor for sustainable livelihoods. More precisely, in Pakistan, the financial security of smallholder farmers is seriously threatened by the occurrence of droughts and floods negatively impacting on agricultural production, with further negative impacts on market price volatility for agriculture commodities, as well as by heavy reliance on agriculture as a single source for income generation. Knowing that reduced financial resources and lack of alternative sources for income generation have been identified as contributors to environmental degradation (Tripathi and Vasan, 2014), two more components, Finance and Knowledge and skills, have been taken into consideration, besides the original ones, for calculating vulnerability more precisely.

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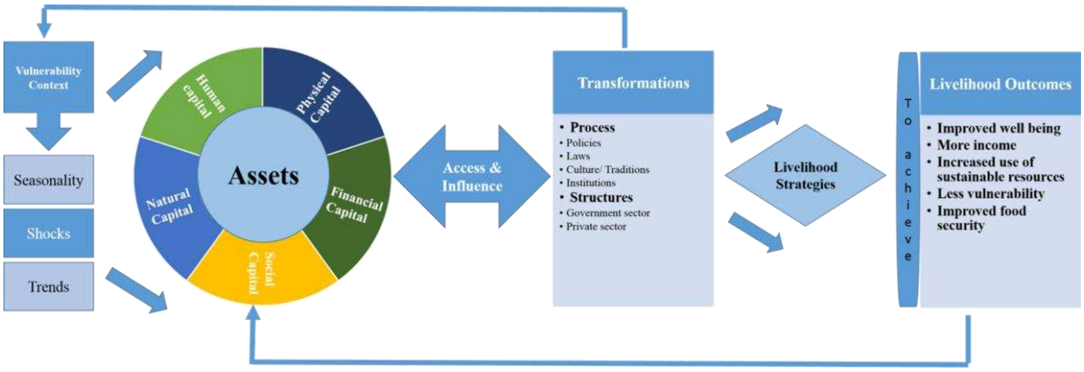

***Figure 1.** Framework for Sustainable livelihoods*

Calculating Livelihood Vulnerability Index

Both internal and external factors relating to smallholder farmer's vulnerability to climate change were taken into consideration for calculating LVI. Balance weighted approach was employed to calculate LVI.

The LVI includes nine essential elements: Socio-Demographic Profile (SDP), Livelihood Strategies (LS), Finance (Fi), Knowledge and Skills (KS), Social Networks (KS), Health (H), Water (W), Food (F), and Natural Vulnerability and Climate Variability (NVCV). Each of these elements is comprised of several sub-components identified in the literature reviewed and presented in *Table 1*. Because of different measuring scale of each of the sub-components, it was first necessary to standardize each of them as an index by using equation (1).

$$Index\ s_d = \frac{S_d - S_{min}}{S_{max} - S_{min}} \qquad \text{(Eq. 1)}$$

Where S_d is the originally observed value of the sub-component for district d, and S_{min} and S_{max} are the minimum and maximum values, respectively, for each sub-component determined using data from both districts. After each sub-component, has been standardized, the obtained values were averaged by using equation (2) to calculate the value of the major component.

$$M_d = \frac{\sum_{i=1}^{n} Index\ s_{d^i}}{n} \qquad \text{(Eq. 2)}$$

Where M_d represents one of the nine major components for district d, denotes the sub-components, indexed by i, that make up each major component, and n is the number of sub-components in each major component. The resulted values of each of the nine principal components were averaged by using the following equation (3) to calculate LVI at the district level.

$$LVI_d = \frac{\sum_{i=1}^{9} w_{Mi}\, M_{di}}{\sum_{i=1}^{9} w_{Mi}} \qquad \text{(Eq. 3)}$$

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Where LVI_d denotes the Livelihood Vulnerability Index for District d, obtained by the weighted average of the nine major components. The weights of each major component, w_{Mi}, were determined by the number of sub-components of each major component and are included to ensure that all sub-components contribute equally to the overall LVI (Sullivan, 2002; Hahn et al., 2009; González Botero et al., 2013). In this study, the LVI is scaled from 0 (least vulnerable) to 1 (most vulnerable).

Calculating Livelihood Vulnerability Index- Intergovernmental Penal on Climate Change

Exposure, Adaptive capacity and Sensitivity are the three contributing factors to livelihood vulnerability, according to IPCC definition of vulnerability to climate change. Organization and arrangements of the nine major components used to calculate LVI-IPCC is presented in *Table 2*. The sub-components in *Table 1*, and equation (1), (2), and (3) were used for the calculation of LVI-IPCC. The LVI–IPCC deviates from the LVI when the major components are combined. The major components were connected according to the categorization scheme in *Table 2*, rather than to merge into the LVI in one step, by using the following equation (4).

$$LVI_d = \frac{\sum_{i=1}^{n} w_{Mi}\ M_{di}}{\sum_{i=1}^{n} w_{Mi}} \qquad \text{(Eq.4)}$$

Where CF_d represents IPCC-defined contributing factor (exposure, adaptive capacity, or sensitivity) for district d, M_{di} denotes major components of the district d indexed by i; w_{Mi} indicates the weight of each major component, and n is the number of major components in each contributing factor. The calculated contributing factors were combined by using the following equation (5)

$$LVI - IPCC_d = (e_d - a_d) \times S_d \qquad \text{(Eq.5)}$$

Where LVI-IPCCd indicates the vulnerability index for district d calculated by using IPCC vulnerability framework, whereas ed, ad, and sd represent the scores of exposure, adaptive capacity and sensitivity for district d respectively. The scale for LVI-IPCC ranges from -1 (least vulnerable) to 1(most vulnerable).

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Table 1. *Major and sub-components with the explanation and source for comprising LVI for two districts*

Major Components	Sub-components	Explanation of Sub-components	Source
Socio Demographic Profile	Dependency ratio	Ratio of the population under 15 and over 65 years of age to the population between 16 and 64 years of age.	(Hahn et al., 2009, González Botero et al., 2013, DHS, 2006)
	Female- headed household	If a male head is away from the home >6 months per year, the female is counted as the head of the household.	(Hahn et al., 2009, González Botero et al., 2013, DHS, 2006)
	Age of the household head	Average age of the household head	(González Botero et al., 2013)
	Households head not attended school	Percentage of households where the head of the household reports that they have attended 0 years of school.	(Hahn et al., 2009, González Botero et al., 2013, DHS, 2006)
	Household family size	Average family members in household	(González Botero et al., 2013)
	Number of years of employment in agriculture	Average agriculture experience of the household head	(González Botero et al., 2013)
Livelihood Strategies	Household family members working outside/ different community	Percentage of households that report at least 1 family member who works outside of the community for their primary work activity	(Hahn et al., 2009, González Botero et al., 2013)
	Household totally dependent on agriculture	Percentage of households that report only agriculture as a source of income.	(González Botero et al., 2013)
	Average agricultural Livelihood diversification index	The inverse of (the number of agricultural livelihood activities +1) reported by a household, e.g., A household that farms, raises animals, and collects natural resources will have a Livelihood, Diversification Index = 1/(3 + 1) = 0.25.	(Hahn et al., 2009, González Botero et al., 2013, DHS, 2006)
	Cultivated Land	Average of the total land cultivated by the Household	(González Botero et al., 2013)
Knowledge and Skills	Farmers not having T. V	Percent of household not having T.V at home	(González Botero et al., 2013)
	Farmers don't share knowledge with each other	Percent of households don't participate in knowledge exchange with others	(González Botero et al., 2013)
	Farmers think they lack in education	Percent of households that think they lack in education	(González Botero et al., 2013)

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Finance	Household having more expense than income	Percent of household having more expenditures than income	(González Botero et al., 2013)
	Household family member work at developed place	Percent of household with any family member working developed place not farming	(González Botero et al., 2013)
	Farmers taken loan	Percent of Household who taken loan in past five years	(Corbett, 1988, Tewari and Bhowmick, 2014)
Food	Farmers depend on land for food	Percentage of households that get their food primarily from their personal farms	(González Botero et al., 2013, Hahn et al., 2009)
	Average Crop Diversity Index	The inverse of (the number of crops grown by a household +1)	(Hahn et al., 2009, Tewari and Bhowmick, 2014, González Botero et al., 2013)
	Farmers that don't save crop	Percentage of households that do not save crops	(Hahn et al., 2009; Tewari and Bhowmick, 2014)
	Farmers that don't save seed	Percentage of households that do not save seed	(Hahn et al., 2009; Tewari and Bhowmick, 2014)
Health	Household family member with chronic diseases	Percentage of households that report at least 1 family member with chronic illness	(Hahn et al., 2009, Tewari and Bhowmick, 2014, DHS, 2006)
	Farmers receiving treatment in hospitals	Percentage of households receiving treatment from government or private hospitals	(Tewari and Bhowmick, 2014, Bhat et al., 2007)
	Time needed to reach hospital	Average time it takes the households to get to the nearest health facility.	(Hahn et al., 2009)
Water	Farmers utilize natural water source	Percentage of households that report a river, lake, or wells as their primary water source.	(Hahn et al., 2009, DHS, 2006)
	Farmers that store water	Percentage of households that store water for household activities and drinking	(Tewari and Bhowmick, 2014)
	Household with depleted water source	Percent of households reported their water source depleted	(González Botero et al., 2013)
	Household without water supply	Percentage of households that report that water is not available at their primary water source everyday	World Bank (1997); (Hahn et al., 2009, González Botero et al., 2013, Tewari and Bhowmick, 2014)
Social networks	Household received help and given help	Help given and received in kind in the past few months	(Hahn et al., 2009, DHS, 2006)

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	Household borrowed or lent money	Percentage of households who borrowed more money than it lent in the past month	World Bank (1998); (Hahn et al., 2009)
	Household not affiliated to any organization	Percent of the households where none of the family member is affiliated with any social institution or organization	(González Botero et al., 2013)
Natural Vulnerability and Climate Variability	Household reported less rain during last few years	Percent of household reported less rain during past few years	(González Botero et al., 2013)
	Household reported more drought	Percent of household reported more drought in past few years	(González Botero et al., 2013)
	Household reported unusual rains	Percent of household reported unusual rains in past few years	(González Botero et al., 2013)
	Household reported increase in temperature	Percent of household reported increase in temperature in past few years	(González Botero et al., 2013)
	Household feel problems in agriculture due to climate change	Percent of household feel problems in agriculture due to climate change	(González Botero et al., 2013)
	Household don't receive warnings regarding severe weather	Percent of Household don't receive warnings regarding severe weather	(González Botero et al., 2013, Hahn et al., 2009, Tewari and Bhowmick, 2014)
	Household change in crop choices/ calendar due to climate change	Percent of household that change their crop choice due to climate change	(González Botero et al., 2013)

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Table 2. *Categorization of the major components for calculation of LVI-IPCC*

Contributing Factors	Major Components
Exposure	Natural vulnerability and climate variability
Adaptive Capacity	Socio demographic profile, Livelihood strategies, Knowledge and skills, Finance, Social networks
Sensitivity	Health, Food, Water

Calculating Livelihood Effect Index

The LEI was determined with the index values of five indicators, namely Human Capital, Natural Capital, Social Capital, Financial Capital and Physical capital. Categorization of the major components into these indicators is shown in *Table 3*. Each value of the five indicators was calculated by using the following equation (6).

$$Cv_d = \frac{\sum_{i=1}^{n} L_{di}}{n} \quad \text{(Eq. 6)}$$

Where Cv_d is the value of each capital of LEI for district d, whereas L_{di} and n represent the value of each effect dimension for capital i of district d and total sub-dimensions forming a capital. After calculating the value of each capital, these values were averaged to compute the LEI of the district by using the equation (7).

$$LEI_d = \frac{\sum_{i=1}^{5} W_i\, Cv_{di}}{\Sigma W_i} \quad \text{(Eq. 7)}$$

Where W_i and Cv_{di} indicate the weight of each capital and value of each capital of district d indexed by i. The LEI scale ranged from 0 (least affected) to 1 (most affected).

Table 3. *Arrangements of major components into different indicators for calculation of LEI*

Indicators	Effect Dimensions
Human Capital	Health, Food, Knowledge and Skills
Social Capital	Socio demographic profile, Social networks
Natural Capital	Water, Natural vulnerability and climate variability
Financial Capital	Finances
Physical Capital	Livelihood strategies

Results

Livelihood Vulnerability Index (LVI)

The average overall indexed values of socio-demographic profile vulnerability index (SDPVI) indicated Chakwal as more vulnerable (0.424) compared to Rawalpindi district (0.356). Among the 120 farmers interviewed in both districts, the average age of the respondents was 51 years. The average percentage of females was 7 and 8, respectively. The average number of years of employment in farming activities was 29 for both districts.

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The average indexed value of the dependency ratio was higher (0.334) for Chakwal compared to Rawalpindi district (0.259). There was a slight difference in the pre-indexed value for female family heads in Chakwal (7%) and Rawalpindi district (8%). The average pre-indexed value for household heads not attended school was higher in Chakwal (70%), as compared to Rawalpindi district (48%). The average pre-indexed value for household family size in both districts was slightly different, with 7.37 persons in Chakwal and 8.07 persons in Rawalpindi district. The average pre-indexed value for the number of years of employment in agriculture was higher in Chakwal (31 years) compared with Rawalpindi District (25 years).

The average overall indexed value of Livelihood strategies vulnerability index (LSVI) was slightly higher for Rawalpindi (0.487) compared with Chakwal district (0.478), whereas a higher percent of farmers in Chakwal (0.71) were solely dependent on agriculture for income generation compared with Rawalpindi district (0.68). The average indexed value of family members employed outside the community was higher for Rawalpindi (0.479) compared to Chakwal district (0.306). The average indexed value for livelihood agricultural diversification index was lower for Rawalpindi (0.167) compared to Chakwal district (0.370). Pre-indexed values showed differences in terms of farmer's family members who are working outside, or in a different community (31 percent in Chakwal and 48 percent in Rawalpindi), percentage of farmers entirely dependent on agriculture (71 percent in Chakwal and 68 percent in Rawalpindi), along with average livelihood diversification index (0.311 in Chakwal and 0.250 in Rawalpindi). While slightly low differences were seen in terms of average cultivated land (3.1 and 3.3 acres in Chakwal and Rawalpindi district, respectively).

The average indexed value for knowledge and skills vulnerability index (KSVI) was slightly higher in Chakwal (0.293) compared with Rawalpindi district (0.246). The average pre-indexed values of KSVI subcomponents indicated very low percentage of farmers not having T.V. (six percent in Chakwal and two percent in Rawalpindi district, respectively), as well as low percentage of farmers who don't share knowledge with each other (four percent in Chakwal and eleven percent in Rawalpindi district, respectively). There was a considerable percentage of farmers who think they lack education (78 percent in Chakwal and 61 percent in Rawalpindi district, respectively).

The average indexed value of FiVI was higher for Rawalpindi (0.593) compared with Chakwal district (0.461). The average pre-indexed values of FiVI indicated a considerable percentage of farmers having more expenses than their income (95.8 percent in Chakwal and 83 percent in Rawalpindi district, respectively) and a comparably low percentage of household family members who worked at developed place (23.6 percent in Chakwal and 56 in Rawalpindi district, respectively. The average pre-indexed value for the percentage of farmers who took loans was considerably higher in Rawalpindi (39 percent) compared with Chakwal district (19 percent).

The average indexed value of the Food Vulnerability Index (FVI) indicated a rather low effect on the vulnerability in Chakwal (0.299) and Rawalpindi (0.349) district. The average pre-indexed value for the percentage of household depending on land for food was higher in Chakwal (71 percent) compared with Rawalpindi (56 percent) district. Similarly, the average indexed value for the same FVI sub-component was higher in Chakwal (0.71) compared with Rawalpindi (0.56) district. The percentage of farmers who don't save crop and seed was low in Chakwal (3.3 and 28.6 percent respectively) as compared to Rawalpindi (8.9 and 58.1 percent respectively) district.

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The average overall indexed value for the health vulnerability index (HVI) value was slightly a higher for Rawalpindi (0.406) compared with Chakwal (0.393) district. The average pre-indexed values of HVI subcomponents indicated higher percentage of household family members with chronic diseases in Chakwal (31 percent) compared with Rawalpindi (21 percent) district, but a comparably lower percentage of farmers receiving treatment in hospitals in Chakwal (37 percent) compared with Rawalpindi (49 percent) district. The average pre-indexed value for the recorded time needed to reach the hospital was higher for Chakwal (150 minutes) compared with Rawalpindi (121 minutes) district.

The average overall indexed value for the water vulnerability index (WVI) was slightly higher for Chakwal (0.453) compared with Rawalpindi (0.396) district. The average pre-indexed values of WVI sub-components indicated considerably low percentage of farmers who utilized natural water source (8 percent in Chakwal and 5 percent in Rawalpindi district, respectively); a moderate percentage of farmers who stored water (29 percent in Chakwal, and 33 percent in Rawalpindi district); and considerably high percentages of farmers who complained that their water source was depleted (48 percent in Chakwal, and 69 percent in Rawalpindi district) and of farmers living without water supply (96 percent in Chakwal, and 51.4 percent in Rawalpindi district).

The average overall indexed value for the social networks vulnerability index (SNVI) was slightly higher for Chakwal (0.519) compared with Rawalpindi (0.476) district. The average pre-indexed values of SNVI sub-components also indicated slight differences in the ratio of farmers not receiving and giving help (1.31 in Chakwal, and 1.35 in Rawalpindi district, respectively), the ratio of farmers who borrowed and lent money (1.5 in Chakwal, and 2 in Rawalpindi district, respectively). The average pre-indexed value for the percentage of farmers not affiliated with any organization was higher in Chakwal (82 percent) compared with Rawalpindi (69 percent) district.

The average overall indexed value for the natural vulnerability and climate variability vulnerability index (NVCVVI) was high for both Chakwal (0.777) and Rawalpindi (0.813) district. The average pre-indexed values of NVCVVI sub-components indicated considerably high percentages of farmers, reporting reduced rainfall during the last year (61 and 81 percent), severe drought (79 and 88 percent), and unusual rain (81 and 85 percent); as well as an increase in temperature (85 and 96 percent) in Chakwal and Rawalpindi district, respectively. Similarly, it was observed that problems in agriculture due to climate change (82 and 93 percent), lack of warnings regarding severe weather (88 and 51 percent), change in crop choices or crop calendar (68 and 75 percent) in Chakwal and Rawalpindi district, respectively. However, these values, except for warning regarding severe weather, were higher for Rawalpindi compared with Chakwal.

The overall average indexed LVI value was higher for Rawalpindi (0.493) compared with Chakwal (0.479) district. The average indexed values of the nine major components of LVI (SDP, LS, KS, Fi, F, H, W, SN and NVCV) for both districts are also illustrated in *Fig. 2* which indicates KSVI as one of the major components with moderate effect on vulnerability. Likewise, *Fig. 2* also indicated FiVI as one of the major components with considerable effect on the vulnerability in both districts.

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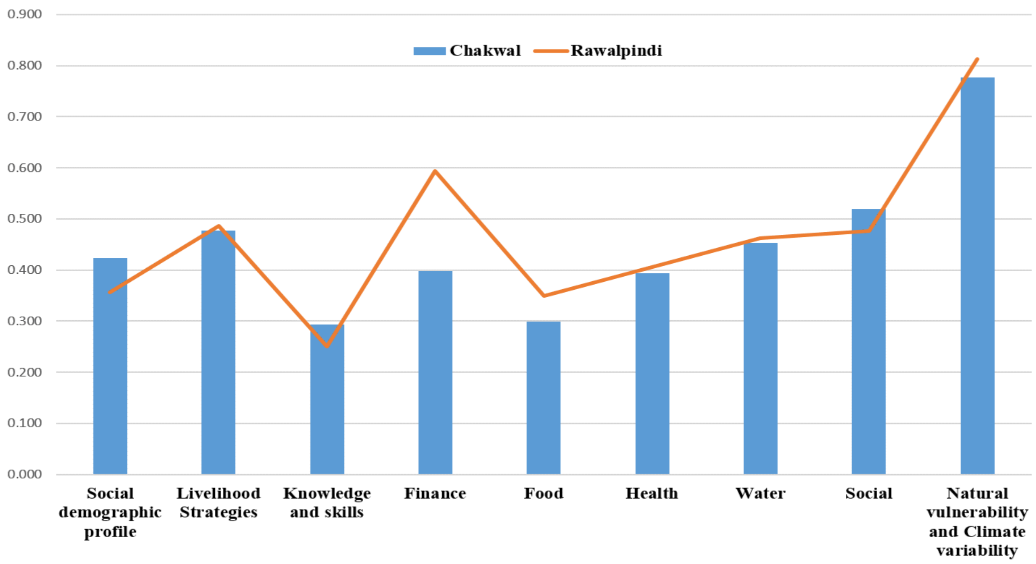

***Figure 2.** Major components of LVI for both districts*

Livelihood vulnerability index- Intergovernmental Panel on Climate Change (LVI-IPCC)

The overall average indexed values of LVI-IPCC were slightly different for Chakwal (0.106) and Rawalpindi (0.089) district. The average indexed values of the three contributing factors (Exposure, Adaptive Capacity, and Sensitivity) indicated slight differences between the two districts in terms of exposure (0.777 and 0.813), adaptive capacity (0.498 and 0.579) and sensitivity (0.381 and 0.382) in Chakwal and Rawalpindi district, respectively. The average indexed values of the three contributing factors, are also illustrated in *Fig. 3*, indicate that district Chakwal has the more adaptive capacity by showing lower vulnerability index compared with Rawalpindi district, whereas people of Rawalpindi are more exposed to climate change compared with Chakwal district. Regarding sensitivity, both districts are shown to be equally sensitive to climate change.

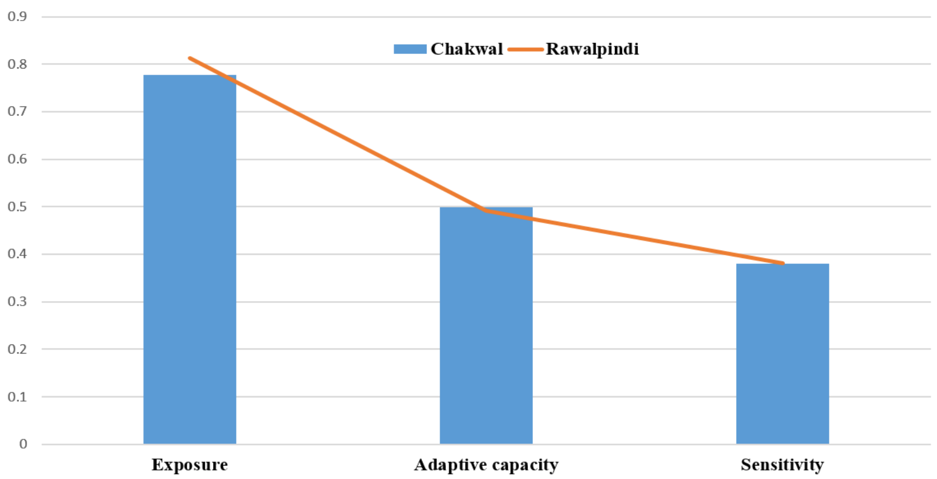

***Figure 3.** Contributing factors of LVI-IPCC for both districts*

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Livelihood Effect index (LEI)

The average overall indexed value of LEI was a little difference for Chakwal (0.454) and Rawalpindi (0.474) district. The major components of LEI are described in *Table 3* and the average pre-indexed, and indexed values of these components are presented in *Table 4*. The arrangement of major components into different indicators used for the calculation of LEI is described in *Table 3*. The average overall indexed value for the human capital was slightly higher in Rawalpindi (0.341) compared with Chakwal (0.326) district. The average overall indexed value for the social capital was slightly higher for Chakwal (0.455) compared with Rawalpindi (0.396) district. The average overall indexed values showed no, or very little, difference for the natural capital (0.659 and 0.685), the financial capital (0.398 and 0.593) and the physical capital (0.478 and 0.487) in both Chakwal and Rawalpindi districts, respectively.

The average overall indexed values for these indicators are also illustrated in *Fig. 4* which showed both districts are more vulnerable to natural capital and less to human capital. Physical capital contributed almost equally to the vulnerability in both districts. People of Rawalpindi district were more vulnerable to financial capital while social capital contributed to vulnerability more in Chakwal compared to Rawalpindi district.

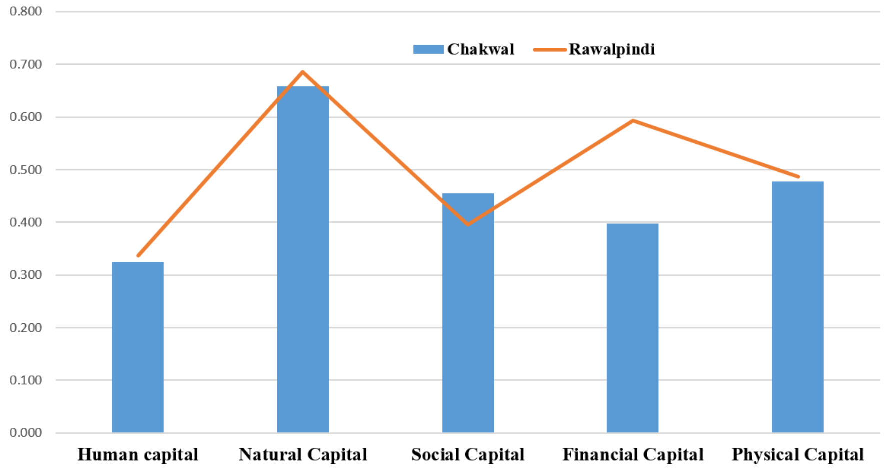

Figure 4. *Vulnerability indicators of LEI for both districts*

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Table 4. *Sub-components, major components and overall LVI, LVI-IPCC, and LEI values for both districts in Pakistan*

Major Components	Sub-components	Units	Pre- indexed values		Indexed values	
			Chakwal	**Rawalpindi**	**Chakwal**	**Rawalpindi**
Socio Demographic Profile	Dependency ratio	Ratio	1.57	0.87	0.334	0.259
	Female headed household	Percent	7	8	0.070	0.080
	Age of the household head	1/ year	0.02	0.02	0.468	0.398
	Households head not attended school	Percent	70	48	0.700	0.480
	Household family size	Average	7.37	8.07	0.421	0.581
	Number of years of employment in agriculture	Average years	31	25.1	0.550	0.338
	SDP Vulnerability index (VI)				0.424	0.356
Livelihood Strategies	Household family members working outside/ different community	Percent	30.61	47.88	0.306	0.479
	Household totally dependent on agriculture	Percent	71	68	0.710	0.680
	Average agricultural Livelihood diversification index	1/ # livelihoods	0.311	0.25	0.370	0.167
	Cultivated Land	Acres	3.1	3.3	0.525	0.622
	LS Vulnerability index (VI)				0.478	0.487
Knowledge and Skills	Farmers not having T. V	Percent	6	2	0.060	0.020
	Farmers don't share knowledge with each other	Percent	4	11	0.040	0.110
	Farmers think they lack in education	Percent	78	61	0.780	0.610
	KS Vulnerability index (VI)				0.293	0.250
Finance	Household having more expense than income	Percent	95.8	83	0.958	0.830
	Household family member work at developed place	Percent	23.6	56	0.236	0.560
	Farmers taken loan	Percent	19	39	0.190	0.390
	Fi Vulnerability index (VI)				0.461	0.593
Food	Farmers depend on land for food	Percent	71	56	0.710	0.560
	Average Crop Diversity Index	1/ types of crops	0.25	0.25	0.167	0.167
	Farmers that don't save crop	Percent	3.3	8.9	0.033	0.089

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	Farmers that don't save seed	Percent	28.6	58.1	0.286	0.581
	F Vulnerability index (VI)				0.299	0.349
Health	Household family member with chronic diseases	Percent	31	21	0.310	0.210
	Farmers receiving treatment in hospitals	Percent	37	49	0.370	0.490
	Time needed to reach hospital	Minutes	150	121	0.500	0.517
	H Vulnerability index (VI)				0.393	0.406
Water	Farmers utilize natural water source	Percent	8	5	0.080	0.050
	Farmers that store water	Percent	29	33	0.290	0.330
	Household with depleted water source	Percent	48	69	0.480	0.690
	Household without water supply	Percent	96	51.4	0.960	0.514
	W Vulnerability index (VI)				0.453	0.396
Social networks	Household received help and given help	Ratio	1.31	1.35	0.437	0.338
	Household borrowed or lent money	Ratio	1.5	2	0.300	0.400
	Household not affiliated to any organization	Percent	82	69	0.820	0.690
	SN Vulnerability index (VI)				0.519	0.476
Natural Vulnerability and Climate Variability	Household reported less rain during last few years	Percent	61	81	0.610	0.810
	Household reported more drought	Percent	79	88	0.790	0.880
	Household reported unusual rains	Percent	81	85	0.810	0.850
	Household reported increase in temperature	Percent	85	96	0.850	0.960
	Household feel problems in agriculture due to climate change	Percent	82	93	0.820	0.930
	Household don't receive warnings regarding severe weather	Percent	88	51	0.880	0.510
	Household change in crop choices/ calendar due to climate change	Percent	68	75	0.680	0.750
	NVCV Vulnerability index (VI)				0.777	0.813
	LVI				0.479	0.493
	LVI-IPCC				0.106	0.123
	LEI				0.454	0.474

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Discussion

Livelihood Vulnerability Index (LVI)

The results for SDPVI indicated Chakwal as more vulnerable compared with Rawalpindi district. The higher average overall indexed value of SDPVI for Chakwal district was mostly due to higher average pre-indexed values for the dependency ratio, household head without education, and many years of employment in agriculture, as well as to the indexed value of the household head. This reads as the farmers in Chakwal district were mostly in the range of age not allowing off-farm employment and self-sufficiency (Hahn et al., 2009; González Botero et al., 2013; DHS, 2006), illiterate, and could only be employed in agriculture, which is known as a sector associated with low income in Pakistan. Among these sub-components, lack of education registered the highest values for both districts and therefore had the highest effect on social vulnerability. This is because the lack of education was found to be associated with less income enhancing and diversification opportunity, generally low levels of income and social exclusion (Van der Berg, 2002; Green, 2006; Gebru and Beyene, 2012). The percentage of female farmers showed little differences in both districts, as females were mostly recorded as household head when husbands or sons lived off-farm for more than six months. The similarity of average pre-indexed values for the percentage of the female household head was due to similar trends of males working outside the community.

The results for LSVI indicated Chakwal and Rawalpindi districts as similarly vulnerable. The close similarity of the average overall indexed values of LSVI for both districts was partially due to similar average pre-indexed values for percentage of farmers entirely dependent on agriculture and surface of the cultivated land for Chakwal and Rawalpindi districts. The remaining part can be explained by lower average pre-indexed value for family members working outside the community but higher average pre-indexed value for agricultural livelihood diversification index in Chakwal compared with Rawalpindi district, which balanced the average overall indexed values of LSVI for both districts. The lower percentage of farmers, exclusively males, working outside the community in Chakwal district was due to the age limitation of the respondents (mostly 16 and 64 years) not allowing them to get employed in off-farm activities. The higher average pre-indexed value for agricultural livelihood diversification index in Chakwal district was mostly due to a higher number of years of employment in agriculture providing the farmers with better skills needed for diversifying agricultural production, on one hand, and the lower income generated from these activities compared with income generated from off-farm activities. Therefore, the respondents from Chakwal district, and to the lower extent from Rawalpindi district, grew crops but also raised livestock and poultry and sold the production surplus on the nearby markets to diversify their income.

The results for KSVI indicated Chakwal as more vulnerable compared with Rawalpindi district. The higher average overall indexed value of KSVI in Chakwal district was mostly due to higher average pre-indexed values for the percentage of farmers who thought they lacked education and the percentage of farmers not having TV. The higher percentage of farmers who thought they lacked education in Chakwal district can be explained by the considerably higher percentage of household heads without education compared with Rawalpindi district. Therefore, the respondents from Chakwal district might have more narrow perspectives on livelihood issues and fewer

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opportunities for improving their living conditions by, for example, adopting new agricultural techniques (Kalinda, 2014; Aasoglenang and Bonye, 2013). There is a need to educate the farmers to cope with the climatic stressors and protect their livelihoods from environmental hazards (Hsueh and Su, 2017).

The results for FiVI indicated Rawalpindi as less vulnerable compared with Chakwal district. The higher average overall indexed value of FiVI for Rawalpindi district was mostly due to the considerably higher pre-indexed values for the percentage of family members working at developed places and the percentage of farmers who took a loan. The higher values were due to a higher percentage of family members working outside the community, and having higher income, but also a higher percentage of farmers taking loans. The social mobility outside the community in Rawalpindi district may have been an attempt to ensure the financial security for the time being and future (Snel and Staring, 2001). One of the main reason for which financial component showed such effect on vulnerability was a much higher percentage of farmers in Chakwal reporting higher expenses than income and taking loans compared with Rawalpindi district. However, the average pre-indexed values for the percentage of farmers taking loans were rather low for both districts, due to the high-interest rates, terms and conditions for receiving loans, and low availability of appropriate institutions in rural areas.

The higher average pre-indexed value for the percentage of households having more expenses than income in Chakwal district was mostly due to the large percentage of the respondents employed in low-income activities, compared with Rawalpindi district. The results for FVI indicated Rawalpindi as more vulnerable compared with Chakwal district. The higher average indexed value of FVI in Rawalpindi district was mostly due to considerably higher average pre-indexed values for the percentage of farmers who did not save crop products and seeds. These higher values can be explained by a higher percentage of farmers employed in off-farm work in Rawalpindi district, on one hand, and by a higher percentage of self-sufficient farmers employed in agriculture in Chakwal district. Therefore, the lower average pre-indexed value for the percentage of farmers totally dependent on land for food in Rawalpindi district. The similarity of average pre-indexed value for average crop diversity index was due to similar crops in both districts.

The results for HVI indicated Chakwal and Rawalpindi district similarly vulnerable. The close similarity of average overall indexed values of HVI for both districts was mostly due to average pre-indexed values for the percentage of household family members with chronic diseases - higher in Chakwal and lower in Rawalpindi district; the percentage of farmers receiving treatment in hospitals - lower in Chakwal and higher in Rawalpindi district; and the time needed to reach the hospital - higher in Chakwal and lower in Rawalpindi district, all contributing to balanced average overall indexed values of HVI for both districts. The slightly lower values for Rawalpindi district were likely due to increased availability of health facilities.

The results for WVI indicated Chakwal as more vulnerable compared with Rawalpindi district. The higher average overall indexed value for WVI in Chakwal district was mostly due to higher average pre-indexed values for percentage of households without water supply, in particular, and, implicitly, the percentage of farmers utilizing natural water sources (i.e. rainwater). The considerably lower corresponding values for Rawalpindi district can be explained by additional income from off-farm activities allowing for investments inadequate farm endowments. Surprisingly, the pre-indexed average values were lower for the percentage of farmers

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storing water and the percentage of households with a depleted water source in Chakwal district. However, no conflict was reported and observed in Chakwal and Rawalpindi districts due to heavy reliance on the rainfall, and, to some extent, the existence of wells ensuring subsistence water supply in the area.

The results for SNVI indicated Chakwal as more vulnerable compared with Rawalpindi district. The higher average overall indexed value of SNVI for Chakwal district was mostly due to considerably higher average pre-indexed value for the percentage of households not affiliated with any organization such as governmental or non-governmental agricultural organizations. These types of organizations are known for their contribution to social capital through knowledge and information transfer, as well as technical support to farmers (Eakin and Bojorquez-Tapia, 2008). The low average pre-indexed values for the ratio of farmers receiving and giving help and the ratio of farmers lending money in both Chakwal and Rawalpindi districts indicated the weakness of social networks. This weakness was found to contribute to increased livelihood vulnerability (Thomas et al., 2005). Therefore, the weakness of social networks, as an aggregate component of LVI, LVI-IPCC, and LEI (*Tables 1, 2 and 3*), is majorly important to reducing livelihood vulnerability and farmers' vulnerability to climate change mostly through enabling the adoption of appropriate mitigating strategies (Hahn et al., 2009). However, the similarly high values for borrowing and lending money components indicate rather stable social networks in both districts.

The results for NVCVVI indicated Rawalpindi as more vulnerable compared with Chakwal district. The higher average overall indexed value of NVCVVI for Rawalpindi district was due to considerably higher average pre-indexed values of all sub-components, except the percentage of farmers not receiving warnings regarding severe weather, compared with Chakwal district. However, all the average pre-indexed value of NVCVVI sub-components were considerably high for both districts. Ahmad et al. (2010) also reported increases in temperature and unusual trend in rainfall for the last five to ten years in both districts. While in another study, Baniasadi et al. (2016) realized that less ground water reserves, low vegetation cover, soil erosion and increased flooding are some of the worst environmental externalities. Nevertheless, these calculated values, based on farmers' inaccurate estimates (Fowler, 2002), need to be compared with values from a natural hazard database at the district level for more accurate prediction of their impact on livelihood vulnerability (Hahn et al., 2009).

The results for the average indexed value of LVI indicated Rawalpindi as slightly more vulnerable concerning livelihood compared with Chakwal district. This was mostly due to higher average indexed values for NVCVVI, lower average indexed values for SNVI and SDPVI. The major components of LVI indicate household characteristics as contributing most to climate change vulnerability in both districts. Among these characteristics, the gender of household head, family size, dependency ratio, and illiteracy level registered values indicating Chakwal district as being socio-demographically highly vulnerable compared with Rawalpindi.

Livelihood Vulnerability Index–Intergovernmental Panel for Climate Change (LVI-IPCC)

The results for the average overall indexed value of LVI-IPCC indicated Chakwal and Rawalpindi district as similarly vulnerable to climate changes. This was due to similar average pre-indexed values for exposure and sensitivity. The higher average pre-indexed value for the adaptive capacity in Rawalpindi district was likely due to reduced

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vulnerability in terms of socio-demographic profile, social networks, and financial situation. Moreover, considerably higher average indexed values for exposure compared with average indexed values for adaptive capacity in both districts indicated their high vulnerability to climate change and their low capacity to adapt to climate changes (González Botero et al., 2013). Moreover, adoption of strategies towards mitigation of climate changes seems to be less possible by the respondents in Chakwal district, with higher illiteracy rate, higher dependency ratio, and higher financial vulnerability, compared with respondents in Rawalpindi district. However, Booysen et al. (2004) argued that if socio-demographic profile changes towards lower dependency ratio and reduced percentage of single-headed households, the adoption of these strategies can increase.

Livelihood Effect Index (LEI)

The results for the average overall indexed value of LEI indicated Chakwal and Rawalpindi districts as similarly vulnerable to livelihood effects. This was mostly due to closely similar average overall indexed values for natural, financial and physical capital. The slightly higher average overall indexed values for human capital in Rawalpindi was mostly due to lower vulnerability in terms of food compared with Chakwal district. The slightly higher average overall indexed values for social capital in Chakwal was mostly due to a higher vulnerability about the socio-demographic profile and social networks.

Natural and financial capital had the highest impact on the livelihoods of smallholder farmers in both Chakwal and Rawalpindi districts. These two types of capitals have been associated with increased livelihood vulnerability (Cobbinah et al., 2013). Surprisingly, the human capital, comprised of health, food, and knowledge and skills components, was associated with the lowest impact on the livelihoods of farmers in both Chakwal and Rawalpindi districts. This could be due to LEI values were based on qualitative estimations, indicating if a person is affected or not by individual factors, without quantifying these effects (Urothody and Larsen, 2010).

Conclusions

Our study has highlighted the problematic state of smallholder farmers in Pakistan, their high exposure to the climatic hazards and the earnest need to decrease both their present and future vulnerability to these dangers. Small landholdings, less innovation, low capitalization and various non-climatic stressors tend to build vulnerability however the strength components, family labor, crop expansion and indigenous knowledge cannot be depreciated. Expanding the profitability of smallholder farming systems is a big challenge that will require a critical and specialized, budgetary and political support at national and global levels. However, these indices will be helpful for planners to assess livelihood vulnerability to climate change sways in the regions of Pakistan and to create projects to reinforce the most vulnerable segments. Replication of this study in the same areas and other locations over the reality of the situation will become obvious eventually how the adaptive capacity, exposure and sensitivity of the districts change as adaptation practices started.

This research work on assessment of smallholder farmers' livelihood vulnerability to climate changes can contribute to developing effective adaptation measures and ensuring sustainable agricultural development. Such research can also provide a scientific basis for the decision-making process and risk analysis of climate change

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impacts in developing countries, where the majority of smallholder farmers, as an important social group worldwide, are located.

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THE PREFERENCES OF THE EUROPEAN BEAVER *CASTOR FIBER* FOR TREES AND SHRUBS IN RIPARIAN ZONES

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Abstract. The aim of this study was to identify selected factors that influence the preferences of European beavers *Castor fiber* for various tree species in three different riparian zones. The study was realized during the November and December of 2015, in 8 research sites located in the immediate vicinity of watercourses. To determine the foraging preferences of beavers, there were analyzed the species and diameter of trees in each riparian zone. Beavers cut down 33.44% trees and partly cut 5.2% trees from 2500 total identified. The results of this study indicate that willows, aspens and alders are most commonly cut by beavers in the fall and winter. In addition to tree species and diameter, beaver foraging preferences are also affected by the distance from the shoreline of a river, watercourse or a water body.
Keywords: *Castor fiber, food, cutting tree*

Introduction

The European beaver *Castor fiber* is a herbivore whose digestive system has adapted to plant foods. The beaver has a relatively long small intestine which is equal to around six-times its body length. The large intestine is also relatively large, and its capacity corresponds to twice the volume of the beaver stomach (Vecherskii et al., 2006). According to Dezhkin et al. (1986), stomach capacity in adult beavers is 350-1400 cm^3, and the digestion process lasts 30-48 hours. In a study conducted in the Voronezh Nature Reserve in Russia, Dezhkin et al. (1986) demonstrated that the daily energy demand of one-year-old beavers reached 1400 calories, whereas two-year-old and older beavers required 1 700 calories per day. The daily energy intake of young animals up to one year of age was estimated at 900 calories.

The annual food intake of an adult European beaver can be as high as 241 kg. In Ural, adult individuals consumed around 111.8 kg of food annually in areas overgrown mainly with birches and alders, and 159.8 kg in sites with a predominance of aspens (Janiszewski and Misiukiewicz, 2012). According to Starinovic (1998), beavers consume around 100 kg of aspens and 230 kg of grass per year. Wood cutting is an important element of beaver behavior (Danilov and Fyodorov, 2015), and it plays a vital role in plant succession and biotope transformations in beaver habitats (Janiszewski et al., 2014, Logofet et al., 2016). Information about beaver foraging preferences can be used to model the utilization of plant foods by beaver families (Nummi, 1989) and to describe environmental changes in riparian zones inhabited by beavers (Rosell et al., 2005). Therefore, the aim of this study was to identify selected factors that influence the preferences of European beavers for various tree species in riparian zones.

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Materials and Methods

We conducted the study in the Polesie National Park in eastern Poland (51°27′19″N; 23°10′24″E) (*Figure 1*). The Park occupies a total area of 9 764 ha, of which 1 211 ha is privately owned.

Figure 1. *Localization of Polesie National Park in Poland*

The Polesie National Park covers wetlands and peat bogs which create an ideal habitat for beavers. The park features lakes (Łukie, Długie, Moszne and Karaśne) which are characterized by varied trophic levels, small area and relatively small depth. The watercourse network in the Polesie National Park is composed of small and highly anthropogenic rivers (Mietiułka, Piwonia and Włodawa). Numerous ditches, channels and canals, which are permanently or seasonally filled with water, constitute an ideal habitat for beavers. Small water bodies, such as former peat pits, marshes and astatic reservoirs, also play an important role in the ecosystem. Around 5% of the park's area is covered with water. The Scots pine *Pinus sylvestris* is the predominant tree species in the Polesie National Park (36.7% of woodland area). Other dominant species that form tree stands are the downy birch *Betula pubescens* (31.9%) and the black alder *Alnus glutinosa* (20.8%) (Radwan, 2002).

We conducted the study in November and December of 2015, in 8 research sites located in the immediate vicinity of watercourses. For the needs of the study, we labeled the research sites with numbers 1 to 8. Every study area was composed of a riparian zone with a length of 200 m and a width of 15 m. We permanently marked the limits of every research site during the study. We found lodges, burrows and dams in all study areas or in their immediate vicinity, which indicated the presence of active beaver families that had been previously monitored by the Park's employees.

The study areas covered various plant formations. Four sites occupied woodlands (sites 1, 2, 3 and 4), two study areas were composed of meadows (sites 5 and 6), and two study areas occupied non-forest tree and shrub communities (sites 7 and 8). We searched for and identified the species of trees and shrubs in the examined study areas (*Table 1*).

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***Table 1.** The number (No.) and percentage (%) of tree species in the analyzed study areas*

Species		Study area								Total
		1	2	3	4	5	6	7	8	
Downy birch *Betula pubescens*	No.	238	11	55	98	63	165	94	0	724
	%	32.9	1.6	7.8	13.6	8.2	22.9	13.0	0	100
Black alder *Alnus glutinosa*	No.	7	359	0	13	60	138	10	132	719
	%	1.0	49.9	0	1.8	8.3	19.2	1.4	18.4	100
Common aspen *Populus tremula*	No.	63	0	5	2	42	20	119	6	257
	%	24.5	0	1.9	0.8	16.4	7.8	46.3	2.3	100
Gray willow *Salix cinerea*	No.	41	0	4	5	36	129	0	0	215
	%	19.1	0	1.9	2.3	16.7	60	0	0	100
White willow *Salix alba*	No.	0	35	0	0	0	0	0	107	142
	%	0	24.6	0	0	0	0	0	75.4	100
Small-leaved lime *Tilia cordata*	No.	0	0	5	16	60	36	0	0	117
	%	0	0	4.2	13.7	51.3	30.8	0	0	100
Pedunculate oak *Quercus robur*	No.	7	5	0	0	2	0	40	38	92
	%	7.6	5.4	0	0	2.2	0	43.5	41.3	100
Alder buckthorn *Frangula alnus*	No.	10	28	0	0	15	3	12	0	68
	%	14.7	41.2	0	0	22.1	4.4	17.6	0	100
Scots pine *Pinus sylvestris*	No.	11	8	0	4	0	0	24	5	52
	%	21.2	15.3	0	7.7	0	0	46.2	9.6	100
Norway maple *Acer platanoides*	No.	0	1	0	0	3	0	0	30	34
	%	0	3.0	0	0	8.8	0	0	88.2	100
Norway spruce *Picea abies*	No.	21	4	0	0	0	0	0	0	25
	%	84.0	26.0	0	0	0	0	0	0	100
Common ash *Fraxinus excelsior*	No.	0	0	0	23	0	0	0	0	23
	%	0	0	0	100	0	0	0	0	100
Common hawthorn *Crataegus mon.*	No.	0	0	0	0	0	4	0	8	12
	%	0	0	0	0	0	33.3	0	66.7	100
European pear *Pyrus communis*	No.	0	0	0	0	0	0	0	10	10
	%	0	0	0	0	0	0	0	100	100
Field elm *Ulmus minor*	No.	0	4	0	0	0	0	0	0	4
	%	0	100	0	0	0	0	0	0	100
Common hornbeam *Carpinus betulus*	No.	1	1	0	0	0	0	0	1	3
	%	33.3	33.3	0	0	0	0	0	33.3	100
Rowan *Sorbus aucupar.*	No.	0	0	0	0	0	0	3	0	3
	%	0	0	0	0	0	0	100	0	100
Total	**No.**	**399**	**456**	**69**	**161**	**281**	**495**	**302**	**337**	**2500**

We determined the number and percentage of each tree species in every study area. We measured the diameter of all tree trunks 20 cm above ground, to the nearest 1 cm, with the use of a caliper. We divided the trees growing in the study areas into three classes based on the degree of damage caused by beavers:

- I – trees cut down by beavers,
- II – partly cut trees,
- 0 – undamaged trees.

We divided every study area into the following zones (belts):

- 5 m from the shoreline (riparian zone A),
- 5 m to 10 m from the shoreline (riparian zone B),
- 10 m to 15 m from the shoreline (riparian zone C).

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To determine the foraging preferences of beavers, we analyzed the species and diameter of trees in each riparian zone. We also described the influence of environmental factors on beaver preferences for the analyzed tree species.

Statistical analyses were processed by on way ANOVA in non-orthogonal design using Tukey HSD test and with Fisher's exact test.

Results

We found a total of 17 tree species in the examined study areas (*Table 1*). The study areas were occupied by a total of 2500 trees. The predominant species were the downy birch (724 trees), black alder (719 trees) and aspen (257 trees).

Beavers cut down 836 trees (33.44%) and partly cut 130 trees (5.2%). We found the highest number of trees (495) in study area No. 8, which accounted for 19.8% of trees in all research sites. We also observed the highest number of cut trees (264) in study area No. 8, which accounted for 31.58% of trees cut in all research sites (*Table 2*).

Table 2. *Percentage (%) of trees representing different damage classes in the particularly study areas*

Species	Class	Study area							
		1	2	3	4	5	6	7	8
Betula pubescens	I	31.3	0	0	0	12.0	18.4	7.37	30.9
	II	0	0	0	0	0	0	42.9	57.1
	0	34.0	18.8	2.2	0	14.4	3.0	8.8	18.8
	Total	32.9	13.0	1.5	0	13.5	7.6	8.7	22.8
Alnus glutinosa	I	0	0	33.3	14.3	0	0	9.5	42.9
	II	0	0	71.2	16.7	3.03	0	6.1	3.0
	0	1.2	1.7	49.3	19.0	1.9	0	8.5	18.5
	Total	1.0	1.4	49.9	18.4	1.8	0	8.3	19.2
Populus tremula	I	36.8	15.4	0	4.3	0	3.4	27.3	12.8
	II	20.0	50.0	0	3.3	3.3	3.3	13.3	6. 7
	0	12.7	78.2	0	0	0.9	0	5. 5	2.7
	Total	24.5	46.3	0	2.3	0. 8	1.9	16.3	7.8
Salix cinerea	I	19.3	0	0	0	2.4	0.9	17.0	60.4
	II	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	6. 7	0	33.3
	Total	19.1	0	0	0	2.3	1.9	16.7	60
Salix alba	I	0	0	15.24	84.76	0	0	0	0
	II	0	0	0	100.0	0	0	0	0
	0	0	0	54.3	45.7	0	0	0	0
	Total	0	0	24.7	75.3	0	0	0	0
Tilia cordata	I	0	0	0	0	4.6	4.6	50.8	40.0
	II	0	0	0	0	16.7	0	83.3	0
	0	0	0	0	0	26.1	4.3	47.8	21.7
	Total	0	0	0	0	13.7	4.3	51.3	30.7
Quercus robur	I	17.6	17.5	29.4	29.4	0	0	5.9	0
	II	0	0	0	100	0	0	0	0
	0	5.7	52.9	0	40	0	0	1.4	0
	Total	7.6	43.5	5.4	41.3	0	0	2.2	0
Frangula alnus	I	41.2		11.8	0	0	0	47.0	0
	II	0	12.5	75.0	0	0	0	12.5	0
	0	7.0	25.6	46.5	0	0	0	14.0	7.0
	Total	14.7	17.6	41.2	0	0	0	22.1	4.4

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Pinus sylvestris	I	0	0	0	0	0	0	0	0
	II	0	100.0	0	0	0	0	0	0
	0	21.6	45.1	15.7	9.8	7.84	0	0	0
	Total	21.2	46.2	15.4	9.6	7.7	0	0	0
Acer platanoides	I	0	0	0	100.0	0	0	0	0
	II	0	0	0	100.0	0	0	0	0
	0	0	0	3.2	87.1	0	0	9.7	0
	Total	0	0	2.9	88.2	0	0	8.8	0
Picea abies	I	0	0	0	0	0	0	0	0
	II	0	0	0	0	0	0	0	0
	0	84.0	0	16.0	0	0	0	0	0
	Total	84.0	0	16.0	0	0	0	0	0
Fraxinus excelsior	I	0	0	0	0	100	0	0	0
	II	0	0	0	0	0	0	0	0
	0	0	0	0	0	100	0	0	0
	Total	0	0	0	0	100	0	0	0
Crataegus mon.	I	0	0	0	83.3	0	0	0	16.7
	II	0	0	0	100.0	0	0	0	0
	0	0	0	0	40.0	0	0	0	60.0
	Total	0	0	0	66.7	0	0	0	33.3
Pyrus communis	I	0	0	0	100.0	0	0	0	0
	II	0	0	0	100.0	0	0	0	0
	0	0	0	0	100.0	0	0	0	0
	Total	0	0	0	100.0	0	0	0	0
Ulmus minor	I	0	0	0	0	0	0	0	0
	II	0	0	0	0	0	0	0	0
	0	0	0	100.0	0	0	0	0	0
	Total	0	0	100.0	0	0	0	0	0
Carpinus betulus	I	50.0	0	50.0	0	0	0	0	0
	II	0	0	0	0	0	0	0	0
	0	0	0	0	100.0	0	0	0	0
	Total	33.3	0	33.3	33.3	0	0	0	0
Sorbus aucuparia	I	0	0	0	0	0	0	0	0
	II	0	0	0	0	0	0	0	0
	0	0	100	0	0	0	0	0	0
	Total	0	100	0	0	0	0	0	0

In the group of identified tree species (*Table 1*), the only species that were not damaged by beavers were Scots pines, common spruces, rowans and field elms (*Table 2*).

Downy birch (*Betula pubescens*) was the predominant tree species in all the study areas, and of the 724 identified trees, 217 trees (29.9%) were cut by beavers (*Table 2*). We found the highest number of birches (238) in study area No. 1, which accounted for 32.87% of birches in all research sites (*Table 2*). We also identified the highest number of cut birches (68) in research site No. 1 which occupied a wooded area. In site No. 1, cut birches accounted for 31.34% of all birches cut by beavers.

Downy birch was most prevalent in riparian zone A where we identified 329 trees (45.4%). We found 185 birches (25.6%) in riparian zone B, and 210 birches (29%) in riparian zone C. The highest number of birches was cut in zone A (151 trees, 45.9%), followed by zone B (38 trees, 20.54%) and zone C (28 trees, 13.33%) (*Tables 5, 6, 7*).

The diameter of birches in the study areas ranged from 3 to 46 cm. The mean diameter of all analyzed birches was 11 cm. The mean diameter of birches was 10.8 cm

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in zone A, 12.5 cm in zone B, and 9.9 cm in zone C. The mean diameter of birches cut by beavers was 6.7 cm.

Table 3. *Percentage (%) of trees representing different damage classes in particularly riparian zones (mean value of 1-8 study areas)*

Species	Class	Riparian zone					
		A		B		C	
		mean	SD	mean	SD	mean	SD
Betula pubescens	I	6,8[a]	0,99	7,2[A]	0,62	5,4[Aa]	0,48
	II	13,8	1,48	16	-	-	-
	0	14,3[A]	0,92	13,9[AB]	1,17	10,5[AB]	0,66
Alnus glutinosa	I	11,0[a]	1,18	7,5a	0,77	10	3,82
	II	11,2	1,19	-	-	-	-
	0	11,5[Aa]	0,98	15,2[a]	1,21	19,5[A]	1,20
Populus tremula	I	15,5[b]	0,86	19,6[ab]	1,04	12,9[a]	1,00
	II	20,2[ab]	0,69	25,1[a]	1,19	29,4[b]	3,82
	0	16,5	0,76	16,4	0,62	16,5	1,24
Salix cinerea	I	3,0	0.34	2,4	0,32	2,6	0,18
	II	0	-	-	-	-	-
	0	8,0	2,65	-	-	-	-
Salix alba	I	8	0,62	8	0,65	25,5	2,69
	II	7	1,70	-	-	-	-
	0	10	0,86	-	-	28	4,27
Tilia cordata	I	4,5[a]	0,62	4,2[a]	0,58	4,8	0,93
	II	10,3	3,69	6,5	1,63	6	
	0	6,9	0,71	7,5[a]	0,74	6,05[a]	0,52
Quercus robur	I	5,3	2,05	8,5	1,21	16	1,48
	II	-		-		18,4	1,99
	0	8,2[A]	0,74	7[a]	0,73	16,9[Aa]	0,94
Frangula alnus	I	4,7	0,53	-		3,8	0,33
	II	8,1	0,78	6	-	-	
	0	4,1[a]	0,25	4	-	5,9[a]	0,64
Pinus sylvestris	I	-		-		-	
	II	4	-	-		-	
	0	19,5	2,63	19[A]	1,82	23,9[A]	1,84

A, B – p ≤0,01
a, b – p ≤0,05

Table 4. *The number and percentage of trees with different diameters cut in the analyzed riparian zones*

Diameter	Riparian zone							
	A		B		C		Total	
	N	%	n	%	n	%	n	%
up to 15 cm	501	90.9	120	79.0	114	85.72	735	87.9
above 15 cm	50	9.1	32	21.0	19	14.28	101	12.1
Total	551	65.91	152	18.18	133	15.91	836	100

Black alder (*Alnus glutinosa*) was represented by 719 trees, of which 63 (8.76%) were cut by beavers. The species was most prevalent (359 trees) in study area No. 3, where it accounted for 49.93% of all black alder trees (*Table 1*). We observed the

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highest number of cut black alders (27) in study area No. 8 which was occupied by non-forest tree and shrub communities. In site No. 8, cut black alders represented 42.86% of all black alders cut by beavers.

Black alder was most prevalent in zone A (587 trees, 81.6%). Zone B was occupied by 81 black alders (11.3%), and zone C – by 51 (7.1%). We found the highest number of cut black alders in zone A (52 trees, 8.9%). Beavers cut 8 black alders in zone B (9.9%) and only 3 trees in zone C (5.9%) (*Tables 5, 6, 7*).

The diameter of black alders in the study areas ranged from 1 to 39 cm. The mean diameter of all black alders was 12.3 cm. The mean diameter of trees was 11.4 cm in zone A, 14.5 cm in zone B, and 19.0 cm in zone C. The mean diameter of all cut black alders was 10.5 cm.

Common aspen (*Populus tremula*) was represented by 257 trees, of which 117 (45.5%) were cut by beavers. The species was most prevalent (119 trees) in study area No. 2, where it accounted for 46.3% of all aspens (*Table 1*). We observed the highest number of cut aspens (43) in research site No. 1 which occupied a woodland. In site No. 1, cut aspens represented 36.75% of all aspens cut by beavers.

Common aspen was most prevalent in zone A (92 trees, 35.8%), followed by zone B (91 trees, 35.4%), and zone C (51 trees, 19.8%). We found the highest number of cut aspens in zone A - 56 trees (60.9%). Beavers cut 33 aspens in zone B (36.3%) and 28 trees in zone C (37.8%) (*Tables 5, 6, 7*).

The diameter of aspens in the study areas ranged from 2 to 40 cm. The mean diameter of all aspens was 17.3 cm. The mean diameter of aspens was 16.7 cm in zone A, 18.6 cm in zone B, and 16.4 cm in zone C. The mean diameter of all cut aspens was 15.8 cm.

Gray willow (*Salix cinerea*) was represented by 215 trees, of which 213 (98.6%) were cut by beavers. The species was most prevalent (129 trees) in study area No. 8, where it accounted for 60% of all gray willows (*Table 1*). We observed the highest number of cut gray willows (128) in research site No. 8 which was occupied by non-forest tree and shrub communities. In site No. 8, cut willows represented 60.38% of all gray willows cut by beavers.

Gray willow was most prevalent in zone A (157 trees, 73%). Zone B was occupied by 18 gray willows (8.4%), and zone C – by 40 (18.6%). We found the highest number of cut gray willows in zone A (154 trees, 98.1%). Beavers cut 18 gray willows in zone B (100%) and 40 trees in zone C (100%) (*Tables 5, 6, 7*).

The diameter of gray willows in the study areas ranged from 1 to 11 cm. The mean diameter of all gray willows was 2.9 cm. The mean diameter of gray willows was 3.1 cm in zone A, 2.4 cm in zone B, and 2.6 cm in zone C. The mean diameter of all cut gray willows was 2.9 cm.

White willow (*Salix alba*) was represented by 142 trees, of which 105 (73.9%) were cut by beavers. The species was most prevalent (107 trees) in study area No. 4, where it accounted for 75.35% of all white willows (*Table 1*). We observed the highest number of cut white willows (89) in research site No. 4 which occupied a woodland. In site No. 4, cut willows represented 84.76% of all white willows cut by beavers.

White willow was most prevalent in zone A (107 trees, 75.4%). Zone B was occupied by 25 white willows (17.6%), and zone C – by 10 (7%). We found the highest number of cut white willows in zone A (74 trees, 69.2%). Beavers cut 25 white willows in zone B (100%) and 6 trees in zone C (60%) (*Tables 5, 6, 7*).

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Table 5. *Percentage (%) of trees representing I class in A riparian zone*

Lp.	Species	(%) class I	(No.) total in zone A	p value 1	2	3	4	5	6	7	8	9
1	*Betula pubescens*	45,90	329	-								
2	*Alnus glutinosa*	8,86	587	**<0,001**	-							
3	*Populus tremula*	60,87	92	0,162	**<0,001**	-						
4	*Salix cinerea*	98,09	157	**<0,001**	**<0,001**	**0,021**	-					
5	*Salix alba*	69,16	107	**0,027**	**<0,001**	0,650	0,075	-				
6	*Tilia cordata*	75,93	54	**0,032**	**<0,001**	0,423	0,293	0,797	-			
7	*Quercus robur*	10,34	29	**0,008**	0,740	**0,002**	**<0,001**	**<0,001**	**<0,001**	-		
8	*Frangula alnus*	14,58	48	**0,003**	0,308	**<0,001**	**<0,001**	**<0,001**	**<0,001**	0,739	-	
9	*Pinus sylvestris*	0,00	5	0,331	1,000	0,159	0,061	0,159	0,076	1,000	1,000	-
10	*Fraxinus excelsior*	63,16	19	0,429	**<0,001**	1,000	0,265	0,846	0,682	**0,008**	**0,007**	0,146

p ≤0,01 – Difference is statistically highly significant

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Table 6. *Percentage (%) of trees representing I class in B riparian zone*

Lp.	Species	(%) class I	(No.) total in zone B	p value 1	2	3	4	5	6	7	8	9	10
1	*Betula pubescens*	20,54	185	-									
2	*Alnus glutinosa*	9,88	81	0,078	-								
3	*Populus tremula*	36,26	91	**0,038**	**0,001**	-							
4	*Salix cinerea*	100,00	18	**<0,001**	**<0,001**	**0,014**	-						
5	*Salix alba*	100,00	25	**<0,001**	**<0,001**	**0,004**	1,000	-					
6	*Tilia cordata*	51,35	37	**0,009**	**<0,001**	0,375	0,135	0,116	-				
7	*Quercus robur*	25,00	16	0,758	0,227	0,783	**0,045**	**0,031**	0,395	-			
8	*Pinus sylvestris*	0,00	9	0,362	1,000	0,111	**0,007**	**0,007**	0,049	0,280	-		
9	*Picea abies*	0,00	21	0,052	0,349	**0,004**	**<0,001**	**<0,001**	**<0,001**	**0,048**	1,000	-	
10	*Crataegus mon.*	62,5	8	0,065	**0,011**	0,349	0,532	0,542	0,756	0,425	0,054	**0,005**	-
11	*Pyrus communis*	0,00	6	0,593	1,000	0,337	**0,029**	**0,028**	0,165	0,542	1,000	1,000	0,128

p ≤0,01 – Difference is statistically highly significant

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Table 7. *Percentage (%) of trees representing I class in C riparian zone*

Lp.	**Species**	**(%) class I**	**(No.) total in zone C**	**p value**								
				1	2	3	4	5	6	7	8	9
1	*Betula pubescens*	13,33	210	-								
2	*Alnus glutinosa*	5,88	51	0,227	-							
3	*Populus tremula*	37,84	74	**<0,001**	**<0,001**	-						
4	*Salix cinerea*	100,00	40	**<0,001**	**<0,001**	**0,002**	-					
5	*Salix alba*	60,00	10	**0,011**	**0,003**	0,392	0,420	-				
6	*Tilia cordata*	19,23	26	0,558	0,134	0,242	**0,001**	0,148	-			
7	*Quercus robur*	21,28	47	0,271	0,075	0,179	**<0,001**	0,101	1,000	-		
8	*Frangula alnus*	55,56	18	**0,002**	**<0,001**	0,482	0,271	1,000	0,134	0,101	-	
9	*Pinus sylvestris*	0,00	38	**0,019**	0,265	**<0,001**	**<0,001**	**<0,001**	**0,015**	**0,005**	**<0,001**	-
10	*Acer platanoides*	2,94	34	0,145	1,000	**0,001**	**<0,001**	**0,003**	0,091	**0,047**	**0,001**	0,479

p ≤0,01 – Difference is statistically highly significant

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The diameter of white willows in the study areas ranged from 2 to 50 cm. The mean diameter of all white willows was 9.7 cm. The mean diameter of white willows was 8.6 cm in zone A, 8.0 cm in zone B, and 26.5 cm in zone C. The mean diameter of all cut white willows was 9.0 cm.

Small-leaved lime (*Tilia cordata*) was represented by 117 trees, of which 65 (55.6%) were cut by beavers. The species was most prevalent (60 trees) in study area No. 7, where it accounted for 51.28% of all limes (*Table 1*). We also observed the highest number of cut limes (33) in research site No. 7 which was occupied by non-forest tree and shrub communities. In site No. 7, cut limes represented 50.77% of all limes cut by beavers.

Small-leaved lime was most prevalent in zone A (54 trees, 46.2%). Zone B was occupied by 37 limes (31.6%), and zone C – by 26 (22.2%). We found the highest number of cut limes in zone A (41 trees, 75.9%). Beavers cut 19 limes in zone B (51.4%) and 5 limes in zone C (19.2%) (*Tables 5, 6, 7*).

The diameter of limes in the study areas ranged from 2 to 27 cm. The mean diameter of all limes was 5.0 cm. The mean diameter of limes was 5.1 cm in zone A, 4.4 cm in zone B, and 5.8 cm in zone C. The mean diameter of all cut limes was 4.4 cm.

Pedunculate oak (*Quercus robur*) was represented by 92 trees, of which 16 (17.4%) were cut by beavers. The species was most prevalent (40 trees) in study area No. 2, where it accounted for 43.48% of all oaks (*Table 1*). We observed the highest number of cut oaks in research sites No. 3 (5) and 4 (5) which occupied woodlands. In those study areas, cut oaks represented 29.41 % of all pedunculate oaks cut by beavers.

Pedunculate oak was most prevalent in zone C (47 trees, 51.1%). Zone A was occupied by 29 oaks (31.5%), and zone B – by 16 (17.4%). We found the highest number of cut oaks in zone C (10 trees, 21.3%). Beavers cut 4 oaks in zone B (25.0%) and only 3 trees in zone C (10.3%) (*Tables 5, 6, 7*).

The diameter of oaks in the study areas ranged from 2 to 36 cm. The mean diameter of all oaks was 13.0 cm. The mean diameter of oaks was 7.9 cm in zone A, 7.4 cm in zone B, and 16.9 cm in zone C. The mean diameter of all cut oaks was 12.1 cm.

Alder buckthorn (*Frangula alnus*) was represented by 68 trees, of which 17 (26.0%) were cut by beavers. The species was most prevalent (28 trees) in study area No. 3, where it accounted for 41.18% of all alder buckthorns (*Table 1*). We observed the highest number of cut alder buckthorns (8) in research site No. 7 which was occupied by non-forest tree and shrub communities. In study area No. 7, cut trees of the discussed species represented 47.06% of all alder buckthorns cut by beavers.

Alder buckthorn was most prevalent in zone A (48 trees, 70.6%); only 2 alder buckthorns (2.9%) were found in zone B, and 18 (26.5%) in zone C. We found the highest number of cut alder buckthorns in zone C (10 trees, 55.6%) and zone A (7 trees, 14.6%) (*Tables 5, 6, 7*).

The diameter of alder buckthorns in the study areas ranged from 1 to 12 cm. The mean diameter of all alder buckthorns was 4.8 cm. The mean diameter of alder buckthorns was 4.8 cm in zone A, 5 cm in zone B, and 4.9 cm in zone C. The mean diameter of all cut alder buckthorns was 4.2 cm.

Scots pine (*Pinus sylvestris*) was represented by 52 trees, of which only 2 (4%) were partially cut by beavers, and no trees were felled. The species was most prevalent (24 trees) in study area No. 2, where it accounted for 46.15% of all pines.

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Scots pine was most prevalent in zone C (38 trees, 73.1%). Zone B was occupied by 9 pines (17.3%), and zone A – by 5 (9.6%). The mean diameter of all pines was 22.4 cm. The mean diameter of pines was 16 cm in zone A, 19 cm in zone B, and 24 cm in zone C.

Norway maple (*Acer platanoides*) was represented by 34 trees, of which only 1 (3.0%) was cut by beavers. The species was most prevalent (30 trees) in study area No. 4, where it accounted for 88.24% of all maples (*Table 1*). Beavers cut only 1 maple in research site No. 4 comprised of wooded areas. Norway maple occupied only riparian zone C.

Common spruce (*Picea abies*) was represented by 25 trees, and it accounted for only 1% of all analyzed tree species. Common spruces were not cut down by beavers.

The remaining tree species (common ash, common hawthorn, European pear, field elm, common hornbeam and rowan) accounted for less than 1% of all identified taxa. Riparian zone A was occupied by a total of 1431 trees, of which 551 (38.5%) were cut and 98 (6.85%) were partly cut by beavers. Riparian zone B was occupied by 503 trees, of which 152 (30.22%) were cut and 17 (3.38%) were partly cut by beavers. Riparian zone C was occupied by 566 trees, of which 133 (23.5%) were cut and 15 (2.65%) were partly cut by beavers (*Tables 5, 6, 7*).

In all study areas, 1427 trees (57.08%) were less than 10 cm in diameter. We determined the number of trees with a diameter smaller than 10 cm at 844 (61.77%) in zone A, at 224 (44.53%) in zone B, and at 319 (56.36%) in zone C. In the total number of trees cut in all study areas, 101 (12.08%) were larger than 15 cm in diameter. We determined the number of cut trees with a diameter larger than 15 cm at 50 (9.1%) in zone A, at 32 (21%) in zone B, and at 19 (14.28%) in zone C (*Table 4*).

Discussion

The results of this study and the findings of other authors clearly indicate that willows, aspens and alders are most commonly cut by beavers in the fall and winter (Ficek, 2003; Vorel et al., 2015; O'Connell et al., 2008; Goryainova et al., 2014). In some studies, beavers chose other species of trees and shrubs, such as *Corylus avellana* (Margaletic et al., 2006). Interestingly, beavers relatively often opted for alder buckthorn (26% trees cut) and pedunculate oak (more than 17% trees cut) in the current study. Gorshkov and Gorshkov (2011) investigated the foraging behavior of the European beaver in the Republic of Tatarstan (European Russia). The authors analyzed 14 000 cut trees and concluded that the foraging preferences of beavers are largely determined by the availability of different tree species in the riparian zone. In the cited study, beavers cut mostly willows (*Salix triandria* and *Salix dasyclados*), which accounted for 92% of all cut trees; whereas willows that were less than 10 cm in diameter accounted for 81% of cut trees. Alder was the second most frequently felled tree, and it accounted for 3% of all cut trees. The cited authors did not report correlations between beaver foraging preferences and the diameter of most tree species, excluding willows and aspens. To cut thicker trees, beavers have to spend more time on land, which increases the risk of a predatory attack. For this reason, beavers select trees not only based on the species, but also based on the diameter of the trunk. In the present study, differences were noted in beavers' preferences for trees that were up to 15 cm in diameter and trees that were larger than 15 in diameter, across the analyzed riparian zones (*Table 4*). On average, nearly 88% (79.0% to 90.9%) of the trees cut by beavers had diameters of up to 15 cm.

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Information about the foraging behavior and preferences of beavers not only expands our knowledge about the species, but also has practical implications. Based on the choices made by beavers, researchers are able to identify the optimal habitats for beavers and estimate the rate at which these animals utilize riparian vegetation. Selective foraging on tree species by beavers in different biotopes is another important consideration. In our study, we observed the highest number of trees damaged by beavers in woodlands and in non-forest tree and shrub communities. Czyżowski et al. (2009) also reported valuable data in a study comparing the foraging preferences of beavers in an urban area (city of Lublin, Poland) and a nature reserve. Their research was conducted near the study areas analyzed in our experiment. In the cited study, white willow and black alder were also most frequently selected by beavers, which can be attributed to the prevalence of these species in the investigated area. In the nature reserve, beavers opted mostly for trees with thinner trunks. The number of damaged trees decreased with an increase in their breast height diameter. The cited authors did not report such a correlation in the analyzed urban area. Beavers also showed a preference for trees representing the most common diameter ranges. The above results suggest that beavers actively select their food sources in natural habitats that are not exposed to external pressures.

In addition to tree species and diameter, beaver foraging preferences are also affected by the distance from the shoreline of a river, watercourse or a water body. Czyżowski et al. (2009) analyzed the depth of incisions made by beavers in trees, subject to the distance from the shoreline. Their findings revealed that beavers most readily foraged for trees and shrubs situated within a distance of 50 m from a watercourse. Trees were most frequently cut in the immediate vicinity of water bodies, but damage to shrubs was noted even within a distance of 80 m from the shore. Shrubs are easy to cut and transport, which prompts beavers to travel greater distances in search of this source of food and building material. The majority of trees cut by beavers were situated within a distance of 10 m from the shoreline (Czyżowski et al., 2009). In the Jamy Forest Division in central Poland, beavers showed a preference for trees with a diameter of up to 15 cm, growing within a distance of 15 m from the shore (Janiszewski et al., 2012). The results of our study and the findings of other authors suggest that beavers concentrate their feeding activity in riparian zones that are 15 m in width. According to Goryainova et al. (2014), beavers generally do not forage further than 50 m away from the shore in early stages of habitat colonization. When local food resources are depleted, the foraging zone can be expanded to 165 m from the shoreline in the absence of pressure from predators. Gorshkov and Gorshkov (2011) demonstrated that the foraging preferences of beavers were more strongly manifested at greater distances from a water body. Aspens were cut at a distance of up to 60 m from the shore, whereas other tree species were damaged mostly within a 10-meter-wide riparian zone.

Nolet et al. (1994) suggested that beaver preferences for tree species could be influenced by the demand for specific nutrients. Beavers could choose hazels and ashes due to their high sodium content, and plum trees and poplars on account of their high phosphorus levels. Therefore, the collection and selection of varied food resources could be a strategy that minimizes the risk of nutrient deficiencies in the European beaver.

Douced and Fryxell (1993) analyzed the relationships between the nutritional value of plant material and the foraging preferences of the Canadian beaver *Castor canadensis*. They found that feeding preferences were not significantly correlated with the energy value or the content of dry matter, energy, total protein or fiber in the analyzed food resources. The cited

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authors suggested that beavers and many other herbivorous vertebrates choose foods that are ample sources of energy. Similar results were reported by Jenkins (1981) who observed that beavers require diverse diets containing various species of plants. When captive beavers were fed monotonous diets composed of aspens only, their body weights decreased. The animals lost more nitrogen than they took in, and their calcium and phosphorus levels were unbalanced. For this reason, free living beavers supplement their diets with aquatic vegetation in the fall and winter when the availability of herbage is limited (Dezhkin et al., 1986). This is particularly important during breeding and nursing when the nitrogen and phosphorus requirements of animals increase six-fold relative to other seasons of the year. Beavers compensate for these demands by foraging on aquatic and herbaceous vegetation (Nolet et al., 1995). In the fall and winter, the animals cut down *Corylus*, *Fraxinus* (Na), *Prunus* and *Populus* (P) to avoid the above macronutrient deficiencies (Nolet et al., 1994).

The biomass of cut trees and shrubs is not fully utilized as food or building material by beavers. On average, beavers eat only round 30% of the collected woody plants, and the remaining resources are left to decompose naturally in the ecosystem. The animals have strong preferences for willows and aspens, which can slow down the succession of these species in floodplains. This process contributes to the growth of other plant species and creates a supportive habitat for aquatic fauna (Janiszewski et al., 2014). Beavers also use shoots and branches from cut trees to build dams. The selection of building materials is determined by the availability of trees in riparian zones, and beavers do not discriminate tree and shrub species based on their suitability for dam construction (Janiszewski et al., 2006).

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MICROBIAL COMMUNITY-LEVEL PHYSIOLOGICAL PROFILES BETWEEN TOKATLI AND SIRÇALI CANYONS, TURKEY

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Abstract. Soil microorganisms are important components of terrestrial ecosystems and play an important role in organic matter fragmentation and nutrient cycling. In our study, microbial diversity of different plant communities and differences in the use of carbon sources in Tokatlı and Sırçalı Canyons were investigated. The canyons were investigated in three stages (tree, shrub and herb). Although there was no significant difference in developmental processes between different vegetation stages, differences between plant communities were significant ($P< 0.05$ and $P< 0.01$). In addition, the groups with the highest variation in carbon sources are *Quercus infectoria* OLIVIER-*Carpinus betulus* L. community in Tokatlı Canyon, and *Quercus pubescens* WILLD-*Pinus nigra* J.F.ARNOLD community in Sırçalı Canyon. The soil carbon content has the most important effect on the diversity of the carbon sources used by microbial communities in both canyons. Carbon source use of microbial communities in soil is affected by dominant species that control environmental factors above the soil.
Keywords: *diversity; plant community; vegetation stages; carbon sources; nutrient cycle*

Introduction

Biodiversity at ecosystem level reveals that not only species or species groups, but also features and processes should be protected (Atik et al., 2010). Soil microorganisms are important components of terrestrial ecosystems. The soil effects nutrient content and primary production. Thus, soil nutrient content and primary production is affected by microorganisms (Rutigliano et al., 2004). Topographic and edaphic factors are significantly influenced by species diversity (Gülsoy and Özkan, 2008). Plants and soil biota have a strong functional relationship as producers and decomposers of terrestrial ecosystems; plants provides organic carbon for soil biota through litter and root exudates, while soil biota decompose organic matter and release mineral nitrogen and phosphorus which are essential for plant growth (Zhang et al., 2010). In general, high plant species richness or functional diversity causes high heterogeneity of resource environments and as a result various microbial groups develop (Sugiyama et al., 2008). Although nitrogen mineralization in soil occurs under control of main soil characteristics, plants also have important functions in this process. Hence, dominant plant species in a region control productivity of an ecosystem by affecting degradation of nitrogen (van Vuuren et al., 1992). Plant species affect soil microbial activity through the quantity and quality of litter they produce. They also affect soil nitrogen conversion rates indirectly. There are important differences in quantity and decomposition characteristics of produced litter between species (Aerts et al., 2006). Also, soil microbial communities play an important to ecological indicator role in maintaining soil fertility and to evaluated soil health (Kumar et al., 2017). Decomposition and mineralization characteristics of organic matter by soil microorganisms are dependent on C / N rate of soil as well as chemical structure of carbon compounds (Krauss et al., 2004).

There are two determinants that shape species diversity in plant communities; species

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pool and ecological connections. Soil organisms such as bacteria, fungi, algae, protozoa and some nematodes play a vital role in preserving soil fertility in organic matter degradation, humus formation and nutrient contents (Smith and Paul, 1990; Lin et al., 2004). Decomposition, cycling and microbial diversity in soil are important indicators of soil microbial function (Fox and Macdonald, 2003). Soil properties are influenced by, for example, soil acidity and nutrient elements of vegetation types (Finzi et al., 1998; van Breemen and Finzi, 1998). Slope differences affect the distribution and development of vegetation and the biological uptake of soil nutrients (Kubota et al., 1998). Soil microorganisms perform in many ecosystem processes, such as nutrient conversion, litter decomposition by regulating the structural and hydrological properties of the soil (Gallardo and Schlesinger, 1994; Kennedy, 1999). Although the effects of soil activity on these processes are well known, we know less about the structure of microbial communities (Derry et al., 1999). Over the last decade, the diversity of soil microbial communities were characterized using commercially available individual Biolog microtiter plates and by production of individual carbon (C) substrates (Insam, 1997). This technique is an ecologically relevant method for measuring microbial biodiversity because it determines the differences in carbon use between communities, which is an important factor. Although the community approach to measure biological diversity is not related to the functioning of the community as a whole, measuring the presence of individuals in the communities can provide more information. Many studies have been carried out on plant diversity in different ecosystems. However, few studies have been undertaken for microbial diversity that contributes to plant diversity for each different habitat (Derry et al., 1999). Canyon ecosystems are special and isolated habitats. Plant diversity of these areas has been studied, but soil microbial diversity and carbon source used has not been studied. This study aimed to determine the effects of biotic factors on diversity of soil microbial communities in isolated canyons and the differences in carbon use between two canyons.

Material and methods

Field study and sampling

The study was carried out in Tokatlı and Sırçalı Canyons, (Tokatlı Canyon: 41° 16′ 0.49″ N - 32° 41′ 0.85″E; Sırçalı Canyon: 41° 15′ 1.20″N - 32° 46′ 9.67″E), mainly covered by enclave mediterranean maquis, rocky slopes, stream coasts and meadows (*Fig. 1*). The vegetation of the area is a mosaic of plant patches of different ages, such as low maquis, high maquis, grassland and rock surface plants (*Fig. 2*). There were a total of 7 different plant communities in canyons, 4 communities in Tokatlı Canyon (*Sedum pallidum*, *Quercus infectoria-Carpinus betulus, Arbutus andrache-Cistus creticus*, *Corylus avellana*) and 3 communities in Sırçalı Canyon (*Fumana aciphylla- Helianthemum nummularium*, *Quercus pubescens-Pinus nigra*, *Juniperus excelsa*). Soil samples (n = 21) were collected in autumn, 2016. Representative surface soil samples (0–10 cm) and total 21 soil sample were taken from plots. All samples were kept at 4 °C from field to laboratory. Soil C was usually oxidized by combustion or wet oxidation and measured by conductivity. Soil pH values were measured in 1:2.5 soil/water extract (Richards, 1954). For electrical conductivity assessment with 1:2.5 ratio, 20 g soil sample was weighed within a conical flask. 50 ml distilled water was added and shaken on horizontal shaker for 15 minutes. Then, the solution in the conical flask was transferred into 100 beaker and left for 15 minutes. At the end of this period, a conductimeter electrode was soaked into clear upper phase and electrical conductivity (EC) value was read when it was stabilized.

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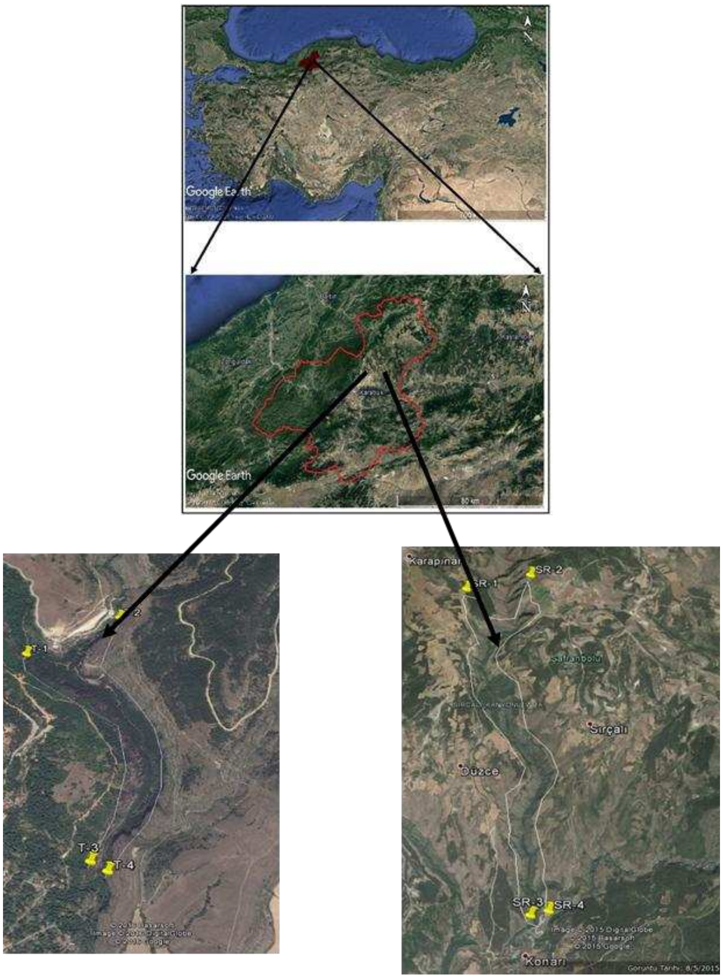

Figure 1. *Location of study area.*

Figure 2. *Habitat photos of study area (a-b: Sırçalı Canyon; c-d: Tokatlı Canyon).*

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Community level physiological profiling (CLPP) analysis

Biolog EcoPlates that are 96-well plates, containing 31 different carbon sources plus a control well, in three replications. The carbon utilization rate was determined with the purple color change of the colorless microorganisms by tetrazolium violet redox. Soil samples (10 g) were shaken for 60 min in 20 ml of a 10 mM Bis–Tris solution (pH 7) and then solutions were inoculated on microplates (100 µl per well) and incubated at 28 °C and allowed to settle for 30 min. Substrate utilization was measured with a spectrophotometer (Multiskan Microplate Photometer–Thermo Fisher Scientific) at 590 nm after 24, 48, 72, 96, 120 and 144 h incubation (Boivin, 2005). ODi (optical density) values obtained at 96 h incubation represented the optimal range of optical density values, so 96 h incubation readings were used for the assessment of microbial functional diversity and determination of carbon sources utilization. Microbial activity in each microplate was determined as average well color development (AWCD). Substrate richness values (R) were calculated as number of wells with color development (Zak et al., 1994). The AWCD was calculated using the equation: $AWCD = \Sigma\ ODi / 31$; the Shannon diversity (H’) was calculated using the equation: $H = -\Sigma\ pi\ (\ln pi)$ (Pi was called proportional color development of the well over total color development of all wells of a plate); and Evenness (E) was calculated using the equation: $E = H / \ln S$ (S = Richness was called number of wells with color development) (Garland, 1997).

Results

AWCD values of metabolized substrates in Biolog EcoPlates were obtained during 144 h for both study areas in two canyons. AWCD generally followed the same pattern of incubation time in both canyons (*Figs. 3* and *4*). There is no difference (*P value* = 0.065) in microbial diversity between different successive processes in Tokatlı Canyon. Significant differences in species diversity (H ') and richness (R) ($P < 0.001$) were found among different communities, while there was no difference between the processes (*P value* = 0.917). The group that caused the difference is *Arbutus andrache* plant community, which is the Mediterranean enclave. In Sırçalı Canyon, there is no significant difference in terms of successions processes and communities. In Tokatlı and Sırçalı canyons, significant differences were found ($P < 0.05$) in succession process levels in terms of AWCD values (*Table 1*). Significant differences ($P < 0.001$) and ($P < 0.05$) were found in R, H and AWCD between different plant communities in Tokatlı Canyon.

Regarding carbon source use in Tokatlı and Sırçalı Canyons, no differences were found in terms of successional processes. However, significant differences were found ($P < 0.0$, $P < 0.05$) for different plant communities. The differences in the use of complex carbons, carboxylic acids, amino acids and carbohydrates in the Tokatlı were found to be highest in *Quercus infectoria - Carpinus betulus* community. The use of amines and phosphate carbon in *Arbutus andrache* community has been found to be at minimum rate. Significant differences were found ($P < 0.01$, $P < 0.05$) in the use of complex carbons, carbonic acids, amino acids and amines as carbon source in Sırçalı Canyon. According to the Tukey’s test, the differences were found to be highest in *Quercus pubescens - Pinus nigra* communities in terms of their complex carbon, amino acids, carboxylic acids and amine amounts (*Table 2* and *Figs. 5, 6*).

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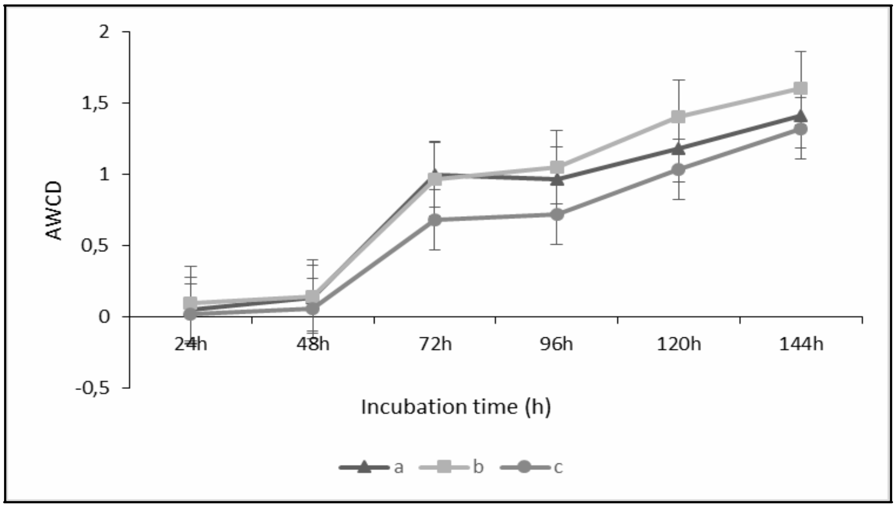

Figure 3. *In Biolog EcoPlates AWCD of metabolized substrates based on 96 h incubation in Tokatlı Canyon.*

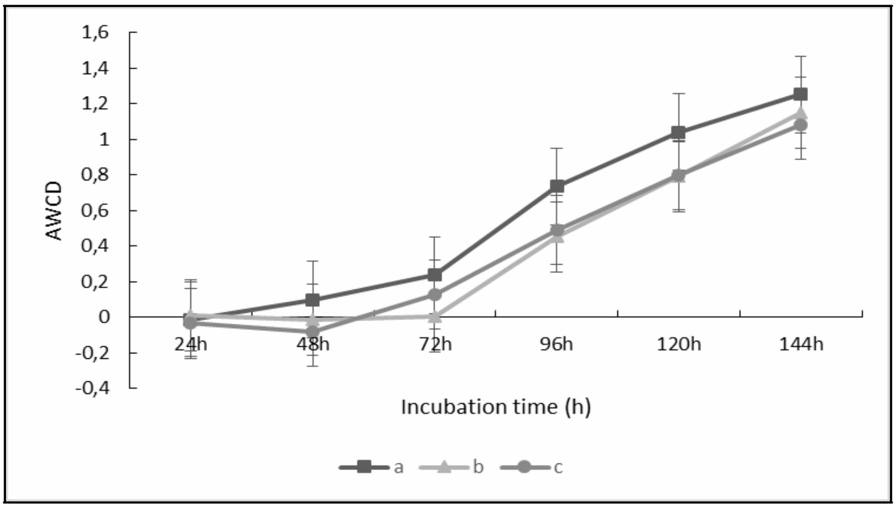

Figure 4. *In Biolog EcoPlates AWCD of metabolized substrates based on 96 h incubation in Sırçalı Canyon.*

Table 1. *Average well-color development (AWCD), richness (R), Shannon–Weaver index (H), Evenness (E) index calculated on carbon substrate use in Biolog EcoPlate (TC: Tokatlı canyon; SC: Sırçalı Canyon). Correlation is significant at the 0.05 and 0.01 level (**P<0.01 and P<0.05).*

	R	H'	E	AWCD
TC	7[b] *0,000****	1.907[b] *0,000****	0,713ns	1,4789[a] *0,015**
SC	0,425ns	0,264ns	0,419ns	1,1798[a] *0,016**

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Table 2. *Carbon usage differences of different plant communities ANOVA test result in two canyons (TC: Tokatlı Canyon; SC: Sırçalı Canyon; Community 2T: Quercus infectoria- Carpinus betulus C Community 3T: Arbutus andrache- Cistus creticus Community 2S: Quercus pubescens- Pinus nigra . Correlation is significant at the 0.05 and 0.01 level (**P< 0.01 and P< 0.05).*

	TC			SC		
Complex carbons	1,9694[a]	*0,024**	Community 2^T	1,4631[a]	*0,041**	Community 2^S
Carboxylic acids	1,5746[a]	*0,002***		1,1915[a]	*0,019**	
Amino acids	1,4470[a]	*0,046**		1,1789[a]	*0,009***	
Carbohydrates	1,8487[a]	*0,040**		1,1223[a]	*0,015**	
Phosphate carbons	1,0237[b]	*0,017**	Community 3^T		*0,080*ns	
Amines	0,7620[b]	*0,003***			*0,101*ns	

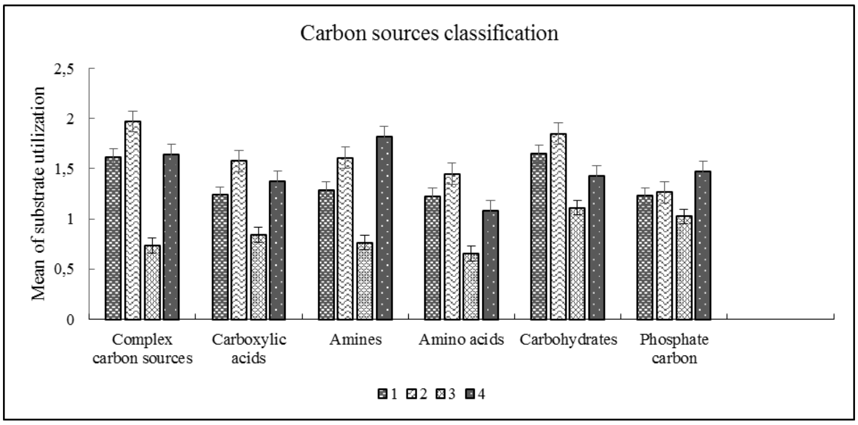

Figure 5. *Mean of substrate utilization carbon substrates from different plant communities* (Community 1: *Sedum pallidum; Community 2: Quercus infectoria-Carpinus betulus; Community 3: Arbutus andrache- Cistus creticus; Community 4: Corylus avellana) in Tokatlı Canyon based on 96-h incubation.*

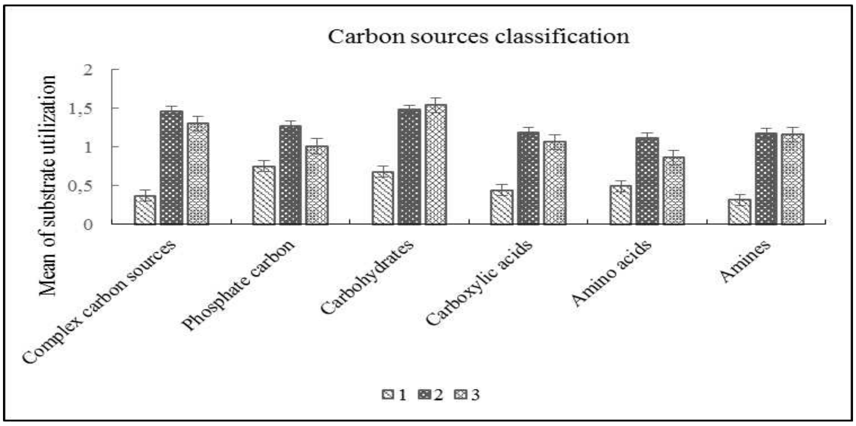

Figure 6. *Mean of substrate utilization carbon substrates from different plant communities (Community 1: Fumana aciphylla- Helianthemum nummularium; Community 2: Quercus pubescens-Pinus nigra; Community 3: Juniperus excelsa) in Sırçalı Canyon based on 96-hincubation.*

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In surface soil samples of both canyons, there are large variations between the carbon sources used by different plant communities on rocky surfaces (*Figs. 7, 8*). This proofs that microbial community varies depending on plant community structure. The first and second factors (PC1 and PC2) accounted for 28.31 and 20.21% of the variance, with a cumulative variance sum of 48.52%, respectively to Tokatlı Canyon. The effectiveness of ecological parameters contributing to the separation of carbon sources used by microbial communities in the field. CCA is indicated by the ordination analysis (*Fig. 9* and *Table 8*).

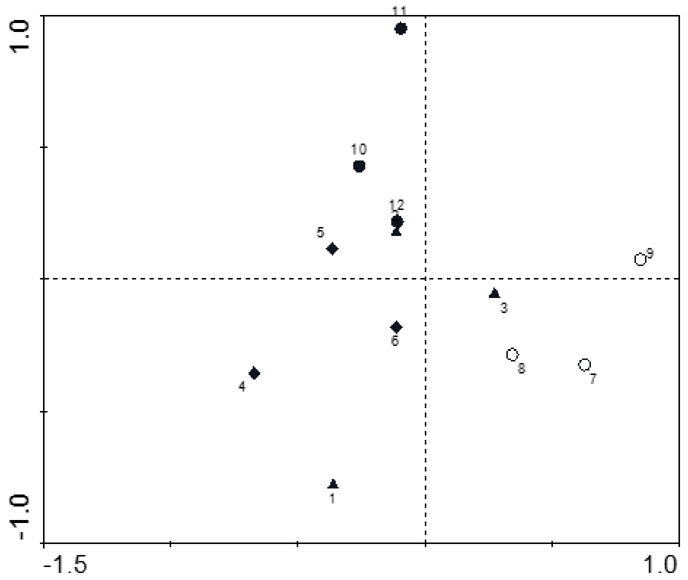

Figure 7. *PCA plot of substrate utilization patterns of microbiological communities estimated using Biolog EcoPlates data in Tokatli Canyon.*

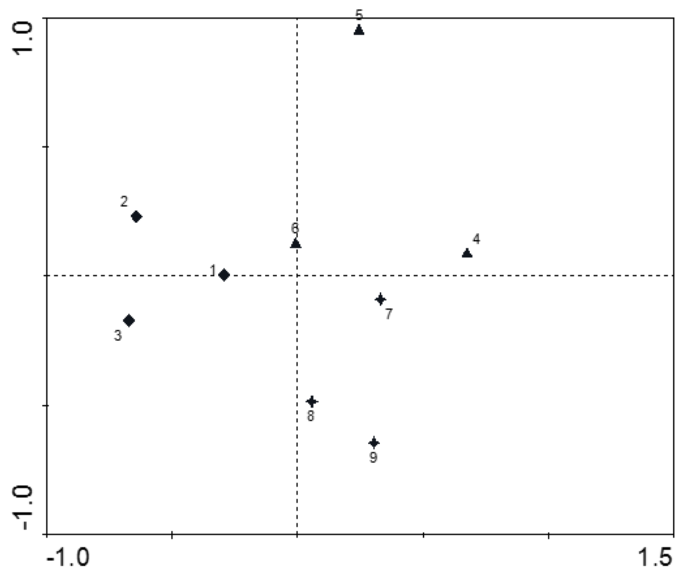

Figure 8. *PCA plot of substrate utilization patterns of microbiological communities estimated using Biolog EcoPlates data in Sircali Canyon.*

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Table 3. *Results for the first and second canonical correlation analysis based on plant andenvironmental data for Tokatli and Sircali Canyons. Significant values were defined in bold.*

	Tokatli		**Sircali**	
	I. axis	**II. axis**	**I. axis**	**II. axis**
Eigenvalues	0.004	0.001	0.043	0.008
Species-Environment correlation	0.771	0.801	1.000	1.000
Percent of restricted cumulative	35.2	46.5	84.6	100
Environmental variables with highest contribution to the axes				
pH	-0.079	0.494	0.296	**0.955****
EC	0.306	**0.734***	**-0.704***	**0.709***
Carbon content	0.256	**-0.566***	**-0.731***	**0.681***

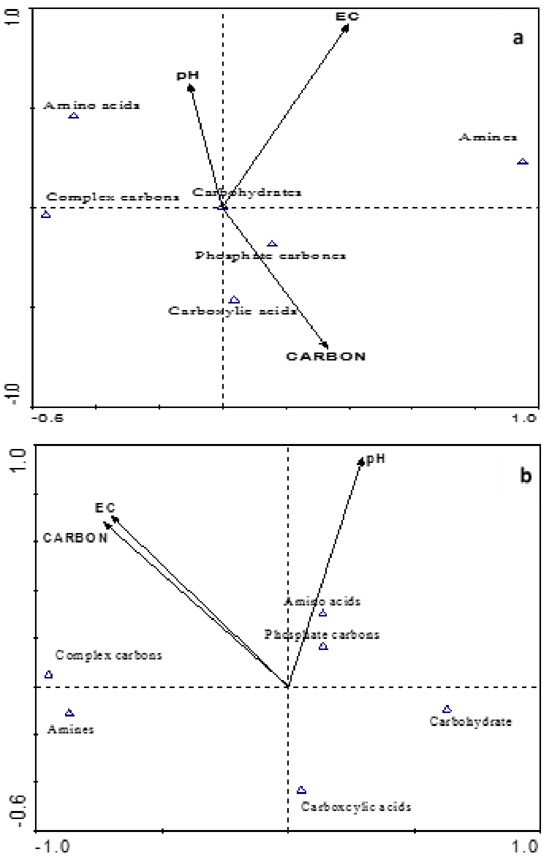

Figure 9. *CCA ordination biplot of Biolog substrates and environmental variables for Sircali and Tokatli Canyons. Triplots indicate the directions and relative importance (arrow lengths) of the three environmental variables. Soil carbon content, soil pH and soil electrical conduktivity (EC). a- Substrates with approximate correlations of $p < 0.05$ to either soil carbon content, electrical conductivity are indicated in Tokatli. b- Substrates with approximate correlations of $p<0.05$ to either carbon contents, soil pH, electrical conductivity are indicated in Sircali.*

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Discussion

As the succession stages progress, symbiotic interactions, mineral cycles, energy and nutrients are increasingly being used effectively. These events lead to the emergence of potential niches. While increasing the number of niches increases species diversity, competition and life span are also increasing. As this situation dominates some living species in the ecosystem, there are emerging elements of balancing species diversity. For this reason, the change of species diversity is not a direct effect of succession but a consequence of its indirect effects. The level of diversity reached determines the energetic relationships in the environment (Odum, 1997). The 95 substrate in the Biolog microplate provides prediction of the relative metabolic potential and responses of the microbiological community (Gomez et al., 2004). Nutrient availability is the main limiting factor for growth in forest ecosystems, and this is highly influenced by the processes in the rhizosphere. In rhizosphere the structure of microbial communities has been shown to vary depending on the type of tree. Microbial growth in soil is limited by carbon sources. As a result of rhizodeposition, carbon in rhizosphere increases microbial activity at high concentrations. Microbial diversity in different plant rhizospheres arise from the diversity of compounds leak from the plants. The data on the interactions between the microbial community and the soil is still insufficient (Graystone and Campbell, 1996). The carbon source utilization diversity of the samples in the Biolog microplate is related to the amount of carbon in the soil, soil pH and electrical conductivity. The soil carbon content is the most important effect on the diversity of the carbon sources used by microbial communities in both canyon areas. These results are similar to Bossio and Scow (1995) and Ritz et al. (1992). Tree layer in Tokatlı Canyon and grass layer in Sırçalı Canyon were responsible for these differences. Long carbon residence times in soil depend on quality and low quantity of litter and lower mineralization rate. Besides, at community level traits that support primary productivity and increase the availability of nutrients are slow degradation and traits that support soil carbon stability. This situation is related with coexistence of species with different growth rates (Hooper et al., 2005). Significant difference was found in terms of carbon use between communities.

Complex carbons, carboxylic acids, amino acids and carbohydrates in Tokatlı canyon were used most effectively by *Quercus infectoria - Carpinus betulus* communities. A mediterranean enclave *Arbutus andrache* community has the lowest use rate in amine and phosphate use as carbon sources. Drought and stress factors reduce the rate of carbon use in mediterranean enclaves while *Quercus - Carpinus* communities form more humid environment and thus affect mineralization level and provide more efficient use of carbon sources. Microbial communities in soil are controlled by climate, edaphic and topographic factors and even by fauna of the area (Zornoza et al., 2009). Dominant plant strategy within an ecosystem depends on the environmental conditions because dominance of species with high growth rates require highly available light and nutrients. Therefore, soil carbon input will be mainly derived from poor-quality litter in biomes with a short growing season and low nutrient availability, whereas primary productivity will be the main driver of soil carbon sequestration in more productive biomes (de Deyn et al., 2008). In this study, factors affecting substrate use in Sırçalı Canyon were pH, EC and soil carbon content. Among these factors, pH caused the most significant effect. Therefore in the area is controlled by nitrate use, because soil pH is influential in mineralization of nitrogen by

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determining the activity of microorganisms that decompose organic matter. In general, nitrate is formed in slightly acidic and slightly alkali (pH 6.0-8.0) soil and an increase in ammonium is seen due to increasing acidity (Doğan, 2012). In Tokatlı Canyon, factors influencing substrate use are EC and soil carbon content, respectively. Microbial markers of soil quality involves various variable parameters at ecosystem, community and population levels. Chemical properties are accepted as essential measurements although they are not sensitive markers as C and N contents (Ros et al., 2006). In our study, the difference in communities affects carbon sources, microbial diversity and richness, while no difference is found in terms of microbial communities for different successive processes (tree, shrub and grassy). Because, in the study areas in two canyons, the formation processes of plant communities on rock surfaces were at the beginning stages, indicating that the development in these areas were at early stages. At canyon surfaces, different plant communities show different carbon use rates. This proofs that microbial communities vary depending on plant community structure. The difference between communities is evidence that the soil microbial content is changing by plant communities settled in the area. Global changes are affecting carbon cycle pathways. Regarding this issue only a few biomes has been studied to date. Therefore, the number of studies on this topic should increase in order to determine the effects of climate change (de Deyn et al., 2008).

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SITUATION AND EVALUATION OF BIOLOGICAL AND CHEMICAL CONTROL APPLICATIONS FOR FOREST IN TURKEY

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Abstract. Pest insect species cause important economic losses in Turkish forests by feeding on various parts of forest trees. To combat them, control methods such as chemical, biotechnical, mechanical and biological applications are used. Among them, biological control is the most important method for the ecological aspect. Technically, the first biological control practice in Turkish forests was launched in the last part of the 1960s. This study aimed to evaluate current control methods for Turkish forestry. For this purpose, biological and chemical control applications in Turkey have been examined and compared. Necessary data were obtained from the 28 Regional Directorates of Forestry in Turkey using a developed data collecting method. Collected data were classified as insect production numbers, control areas and expenditures according to years. Subsequently, the total and unit costs were calculated and the gain and loss amounts obtained were estimated. Based on the comparison between chemical and biological control methods, chemical control is approximately 1.4 times more expensive than biological control in Turkey. This indicates that chemical control is causing economic loss in addition to ecological damages. In conclusion, the primary benefit of biological control is that it restores ecological balance and thus ensures the continuity of ecosystem services. The secondary benefit is savings from lower costs. Therefore, the use of biological control to mitigate damage from insects in forest ecosystems is important for the ecological and economic sustainability of forest ecosystems. To this end, predatory species such as *Rhizophagus grandis* Gyll, *Rhizophagus depressus* (F.), *Formica rufa* L., *Calosoma sycophanta* (L.) and *Thanasimus formicarius* (L.) have been used for biological control operations in Turkish forestry in recent years.
Keywords: *ecological-economic sustainability, pest control, natural balance, forest ecosystem*

Introduction

The forest ecosystem is the most fundamental and indispensible element of human life on earth. The history of utilization of forest resources is as old as humanity. Ecosytem services like carbon sequestration, non wood forest products, biodiversity and other attemps to adress climate change have vital importance for humankind. For the sustainable management of an ecosystem, the factors that compose the ecosystem should also be in balance. From this perspective, insects that had a natural role in a forest ecosystem may proliferate excessively due to various reasons, including human impact. It is of great importance to restore the forest ecosystem back to its natural balance. In order to do so, it is necessary to identify the factors and take the required measures. It is imperative to protect and ensure the sustainability of this vital and indispensible element of life (Tolunay and Türkoğlu, 2014). Given that forest ecosystems constitute a whole with their specific soil structures, plant communities and other organisms, it is important to protect and develop forest ecosystems with a view to ensuring their long term sustainability (Akyol and Tolunay, 2014).

Insects are among the most crucial threats to forests. In recent years, biological control methods that do not harm trees were proposed and put in practice to adress the disadvantages of chemical methods. The chemical insecticides that have negative effects and are dangerous for human health also lead to an insect resistance problem, which increases the pesticide cost because new compounds are needed. Today, it is understood that insecticides cannot provide a permanent solution for the control of insects. Biological control in which natural enemies of the pest insects are used avoids the negative consequences of chemical control on forest ecosystems.

Biological control can also be used to restore the natural balance from the human health perspective. The general principle of biological control is to protect, strengthen the natural enemy populations that have an impact on pest insects, and supplement them with imported species when needed. Technically, the first biological control practice in Turkish forests was launched in 1967 when *Formica rufa* was transplanted from the forest stands in North Anatolia to the Mediterranean region (Oğurlu, 2000). Predatory species such as *Rhizophagus grandis* Gyll, *R. depressus* (F.), *Formica rufa* L., *Calosoma sycophanta* (L.), *Thanasimus formicarius* (L.), as well as insectivore birds are used in biological control operations in Turkish forests; the rearing of predatory species was given priority after 2000s.

The ecological effects and consequences of insect control have been studied in the literature (Greatheat, 1976; Dijken, 1986; Brower, 1991; Van Lenteren, 2003; Orr, 2009; Uygun et al., 2010). However, there are only a limited number of studies that explore the economic aspects of insect control (Bokonon-Ganta, 2002; Born et al., 2005; Mc Fdyent, 2008; Fowler et al., 2016). In particular, there are very few studies in Turkey that explore the economic aspects of insect control methods. Existing studies mainly focus on the damage caused by insects to raw wood material and the associated economic loss. For example, Güngör and Daşdemir (2014) analysed the economic effects of *Pityokteines curvidens* Germ. on the sales of fir wood.

The native tree species in Turkish forests have lost many of their characteristics, especially in some regions, due to long years of irregular utilization and inappropriate interventions (Atmış et al., 2007). Besides raw wood materials, forests play an important role protecting biological diversity. Harmful insect species that lead to considerable economic losses in Turkish forests are controlled through various methods; the General Directorate of Forestry (GDF) spends a significant amount of money for that purpose. Therefore, this study evaluated the current status of biological control practices in Turkish forests and compared it with chemical controls method from the ecological-economical perspective.

Materials and methods

In this study, the insect control methods in Turkish forests were evaluated in general terms as a first step. Secondly, biological control methods and practices were examined. Finally, biological and chemical control methods were compared and analysed from an economical perspective. Data from 2004 to 2014 relating to these control methods, practices and economic expenditure in Turkey were evaluated. As it is difficult to express the benefits of sustainability of ecosystem services in insect control areas in economic and monetary terms, the costs of control methods were highlighted (Pak et al., 2010; Deniz and Ok, 2015).

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Data were collected separately from 28 Regional Directorates of Forestry across Turkey in 2015 and 2016. The collected data included information about the size and cost of areas where chemical methods were applied, successful and failed cases for chemical and biological control methods and amounts of predatory species provided. Total and unit costs were estimated, and the amount of profit and loss was determined. Previous costs were converted to 2015 values using the Domestic Producer Price Index (D-PPI). Moreover, the Turkish Central Bank's data were used for international audiences and the unit costs were converted to USD. In general economic evaluations, all costs were taken into account, irrespective of the success status of insect control efforts in the relevant areas. However, the areas where the methods were successful or failed were taken into account when comparing the biological and chemical control methods; not only the economy of a method but also its success at the intended level is important. Therefore, biological and chemical control methods were evaluated from the perspective of cost minimization and cost saving regardless of their ecological impacts.

Results and discussion

Biological control practices in Turkish forests

The insect control methods that have been implemented in Turkey include chemical control with insecticides, biotechnical control with pheromone and pheromone traps, and mechanical control by way of collecting nest and egg batches of pine processionary moths (*Thaumetopoea wilkinsoni and T. pityocampa*) or trap trees for bark beetles (GDF, 2015). However, biological control methods appear to be preferred over these other control methods in recent years (*Fig. 1*); chemical control methods that cause harm to forests, organisms and people are largely abandoned.

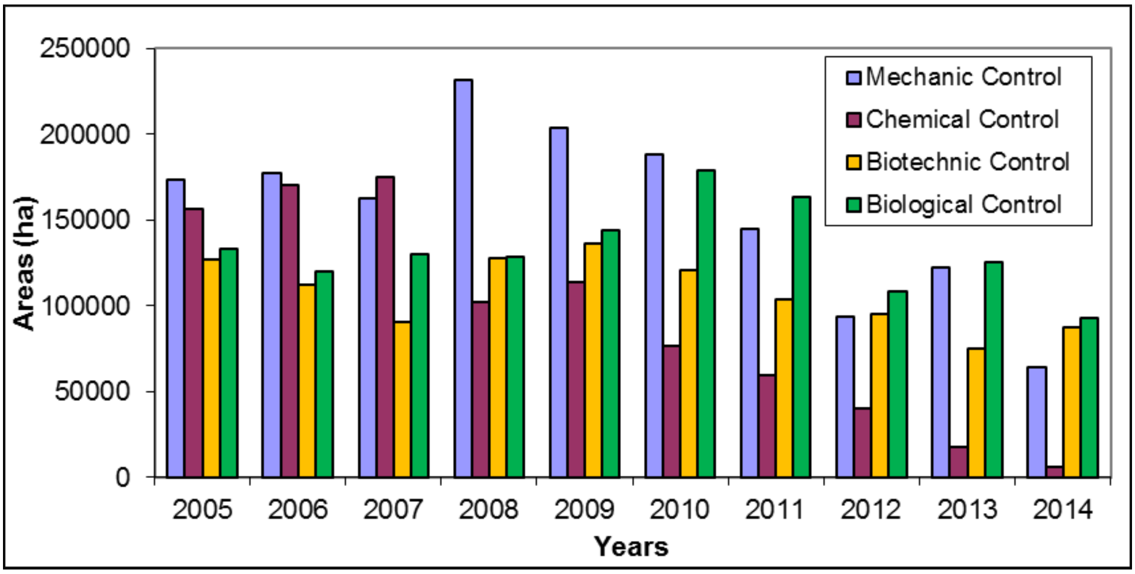

Figure 1. *Distribution of control methods by areas in Turkish forests (2005-2014)*

Formica rufa (Hymenoptera, Formicidae) is one of the important natural enemies used against the pest insects in Turkish forests. They can feed above and below the soil, and can reach the tops of the highest trees in an area with a radius of 25-100 m

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depending on the individual. It was found that a *F. rufa* colony can kill 100.000 insects within 24 hours (Avcı et al., 2000). Therefore, four proper nests can control an area of one hectare. For that reason, it is important to use *F. rufa* ants for biological control with a view to protecting the health of forests and ensuring the continuity of the ecological balance (Oğurlu, 2000; Avcı et al., 2000). *F. rufa* is naturally distributed intensively in the West Black Sea region located in the northwest of Turkey, most parts of the Black Sea region, some parts of the Marmara, Central Anatolia, and the Inner Aegean region in the west. The forest in Senirkent-Kapıdağ of Isparta province in the southwest of the country constitutes the southern boundary of the distribution area of the species. Their presence in Turkey was identified at an elevation of 950-2000 m and mainly in *Pinus sylvestris* forests (Avcı et al., 2000). The transplantation of *F. rufa* nests started in the 2000s, and increased significantly; successful practices were observed (*Fig. 2*).

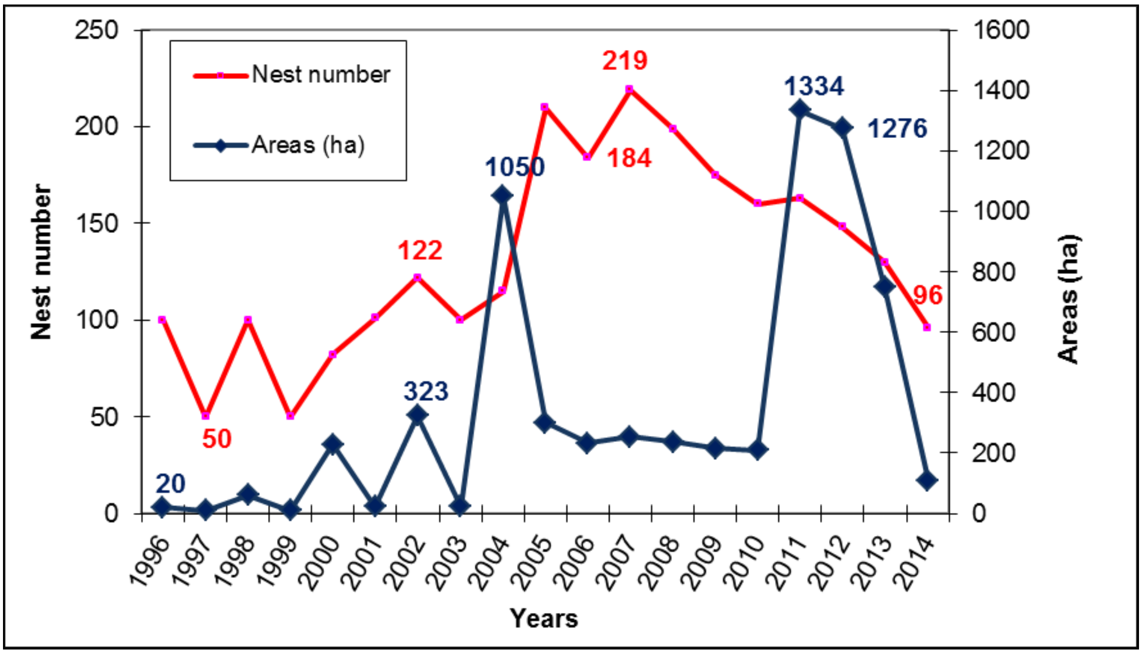

Figure 2. *Transplantation of Formica rufa nests (1996-2014)*

Calosoma sycophanta (L.) is on one of the most important predatory species for the forestry office in Turkey, produced in biological control laboratories established in different parts of the country and used against pine processionary moths (PPM). Mechanical control of PPM is expensive, while chemical control is known to have an adverse effect on the environment. In contrast, the adults and larvae of *C. sycophanta* that have a lifespan of 3–4 years affect the larvae and pupa of PPM *T. wilkinsoni*, which is very important for biological control. PPM causes physiological and primary damage on *Pinus brutia*, *P. sylvestris*, *P. pinea, P. halepensis* and *P.nigra* trees in Turkey. The adults of *C. sycophanta* can kill around 5-10 larvae per day. Considering that the adults can eat 7 PPM a day on average, an adult can kill 280 larvae a year on average, and around 900-1000 larvae on average throughout its life (Kanat et al., 2005; Anonymous, 2006). *C. sycophanta* is also a predator of *Lymantria dispar* and *Euproctis chrysorrhoea* that cause damage to beech, willow, linden and fruit trees. *C. sycophanta* feeds on the

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larvae and pupa of the insects. GDF started rearing this species more intensively especially after 2004, in its biological control laboratories. The method of transplanting the larvae of *C. sycophanta* to the areas where PPM are distributed intensively has been very successful (*Fig. 3*).

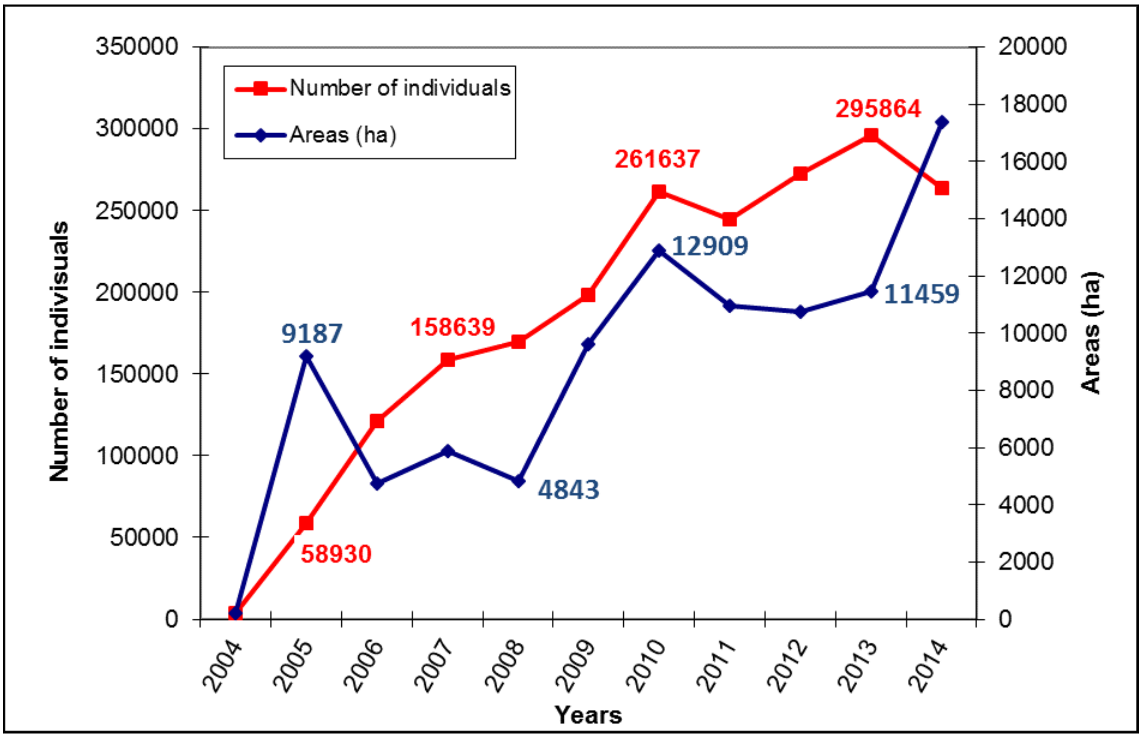

Figure 3. *Rearing and application areas of Calosoma sycophanta (2004-2014)*

One of the most important predators for bark beetle species in Turkey is *Thanasimus formicarius* (L.) (Coleoptera, Cleridae). This predator feeds on the adults, caterpillars and pupa of the bark beetles (*Ips typographus, Pityokteines curvidens, I. sexdentatus, Orthotomicus erosus* and *Tomicus minor* etc.) that cause damage to all coniferous forests (pine, fir, spruce) (Anonymous, 2006). *T. formicarius* is an important natural enemy, as it moves fast and thus can capture and kill the bark beetles. *T. formicarius* produces one generation per year in the East Black Sea region (180–1800 m) located in the northeast of Turkey and the Erzurum Sarıkamış location (2000-2400 m) in the east. The flying period of the insect is March-April at 180 m in the East Black Sea region, and extends from May to September at an elevation of 2000 m in Erzurum Sarıkamış. Studies show that *T. formicarius* can consume around 10 adult bark beetles per day and around 300 adult bark beetles throughout its lifespan (Akbulut et al., 2005). *T. formicarius* is a very important predator of *I. typographus* by feeding on its larvae, pupa and adults. *T. formicarius* is considered to be an important factor for the stabilization of *I. typographus* populations. Studies have reported that 81% of *I. typographus* in West Germany and 53% in Switzerland die in the logs where it coexists with *T. formicarius*. The number of *T. piniperda* also decreased by 92% in the environment where it coexists with *T. formicarius* (Yüksel et al., 2001). Akbulut et al. (2005) reported that *T. formicarius* had an effect on all bark beetles, though limited, and it had the most successful proliferation and attack on scotch pine (*Pinus sylvestris*) and oriental spruce

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(*Picea orientalis*) forests. *T. formicarius* is found intensively on the main galleries of *I. sexdentatus* in scotch pine forests, and prefers primarily *I. typographus* in the oriental spruce forests and then *I. sexdentatus*. It is determined that it prefers *P. curvidens* (%16.29) as a main target (Akbulut et al., 2005). *T. formicarius* is another important species farmed by GDF. This species is currently used in the field against bark beetles to a significant extent (*Fig. 4*).

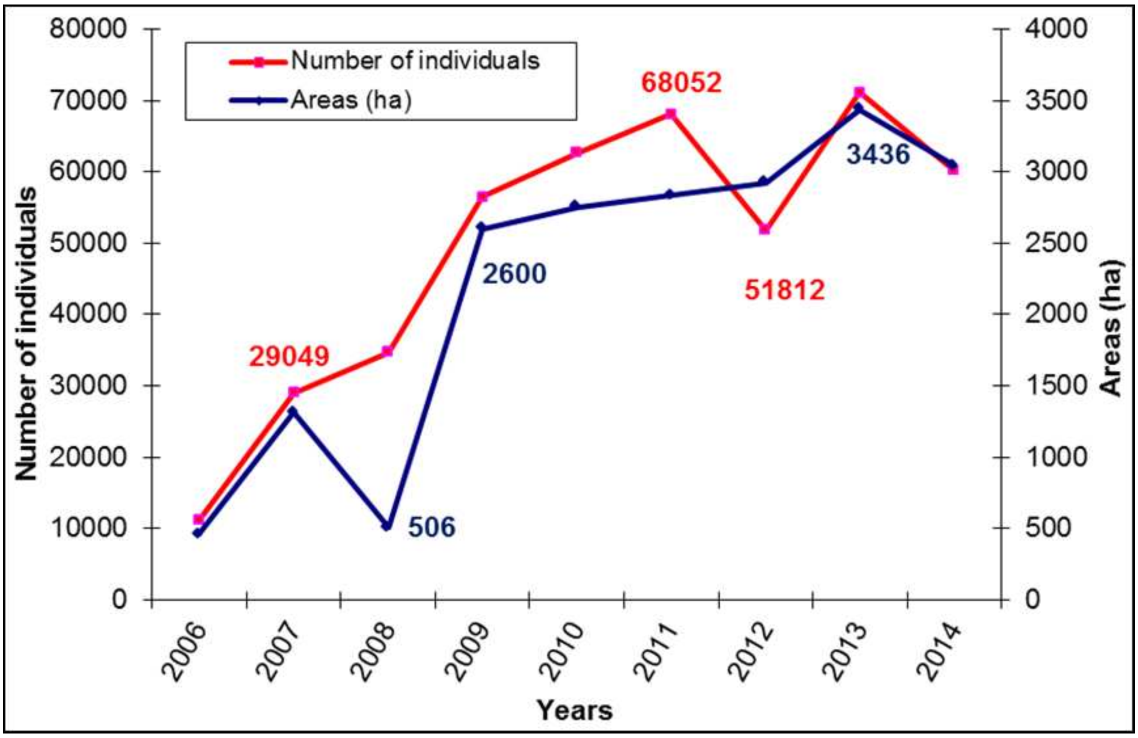

Figure 4. *Thanasimus formicarius rearing and application areas (2006-2014)*

Rhizophagus grandis Gyll. (Coleoptera, Rhizophagidae) is distributed in the Central and Northern European areas, Baltic countries and Siberia. In Turkey, *R. grandis* is an effective natural enemy of *Dendroctonus micans* Kug. which causes extensive damage to oriental spruce forests in an area of 75.000 hectares in Artvin and Posof located in the northeast. Bergmüller (1903) first reported that *R. grandis* was an important species in preventing the *D. micans* epidemics in Europe. Great achievements have been obtained with regard to the control of these insects in Turkey and many European countries. The epidemic of *D. micans* was mitigated with *R. grandis* by around 80–85% (Yüksel and Koçyiğit, 2001). Özcan et al. (2005) found that *R. grandis* could control *D. micans* by 78%.

R. grandis is being reared for the first time in Turkey, and has been used successfully for many years against *D. micans* that causes damage to oriental spruce (*Picea orientalis*) forests. In those treated areas, *D. micans* is no longer a significant threat and the natural balance has been restored in the spruce forests. As a consequence, the rearing of *R. grandis* has declined since 2009 (*Fig. 5*).

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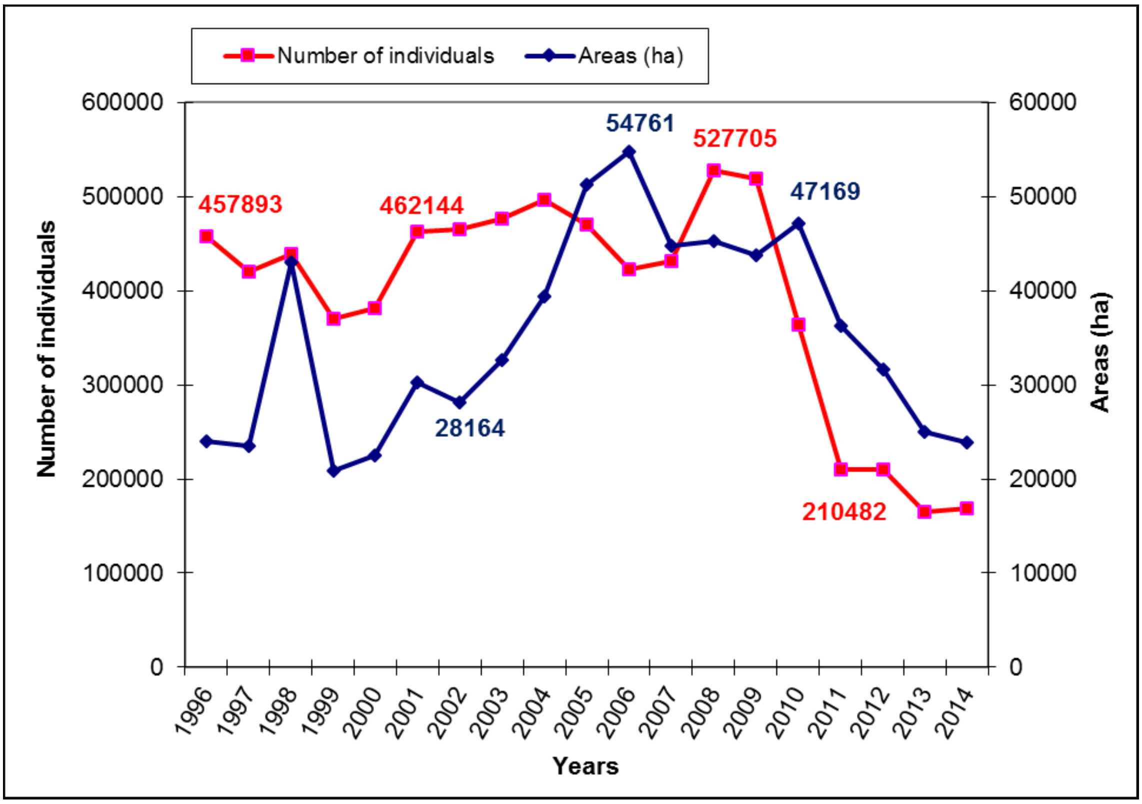

Figure 5. *Rhizophagus grandis rearing and application areas (1996-2014)*

Rhizophagus depressus (F.) (Coleoptera, Rhizophagidae) is another important predatory of bark beetles in Turkey. This species is distributed in the East Black Sea region at an elevation of 1000-2000 m, in Erzurum Sarıkamış at an elevation of 2000-2400 m., and in Bolu and Aladağ at an elevation of 1000-1700 m. with 2 generations per year. *R. depressus* attacks the nests of *I. sexdentatus* (58.43%), *I. acuminatus* (38.13%), *T.minör* (30.49%) and *T. piniperda* (29.52%) in *Pinus sylvestris* forests. The most important results of *R. depressus* were obtained against the insects that damaged *P. sylvestris* forests.

Particularly, *R. depressus* has been reported to have high efficacy on the populations of *I. sexdentatus* in Sarıkamış. The density of this predatory species in the nests of bark beetles in Sarıkamış is at minimum 31.89% and decreased the harmful population in *T. piniperda* nests by around 41%. The harmful insect species it prefers in the spruce forests are *Pityokteines spinidens*, *O. erosus*, and *I. typographus* (Yüksel et al., 2005). Meydan et al, (2005) reported that the density of *R. depressus* is 39% in the nests of *T. minor*, 33% in the nests of *T. piniperda*, 25% in the nests of *I. acuminatus* and 57% in the nests of *I. sexdentatus*. In the study, it was found to prefer mainly *I. sexdentatus*. While *R. depressus* was reared in the biological control labs of GDF and transplanted to nature in order to prevent the epidemics caused by this bark beetle in Turkey, a more effective species, *Thanasimus formicarius,* has been reared predominantly in recent years. Therefore, the quantity of *R. depressus* insects reared has declined significantly since 2013 (*Fig. 6*).

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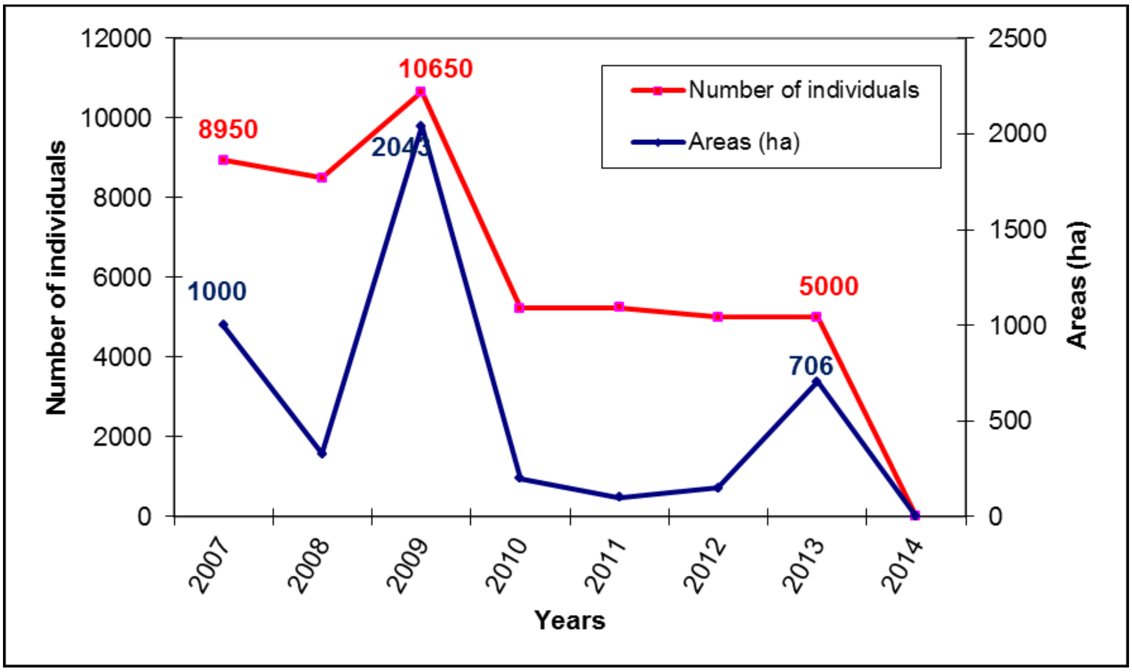

Figure 6. *Rhizophagus depressus rearing and application areas (2007-2014)*

Economic evaluation of biological and chemical control practices in Turkish forests

An analysis of the cost of insect control practices performed by the GDF reveals that mechanical control is one of the most expensive (*Fig. 7*).

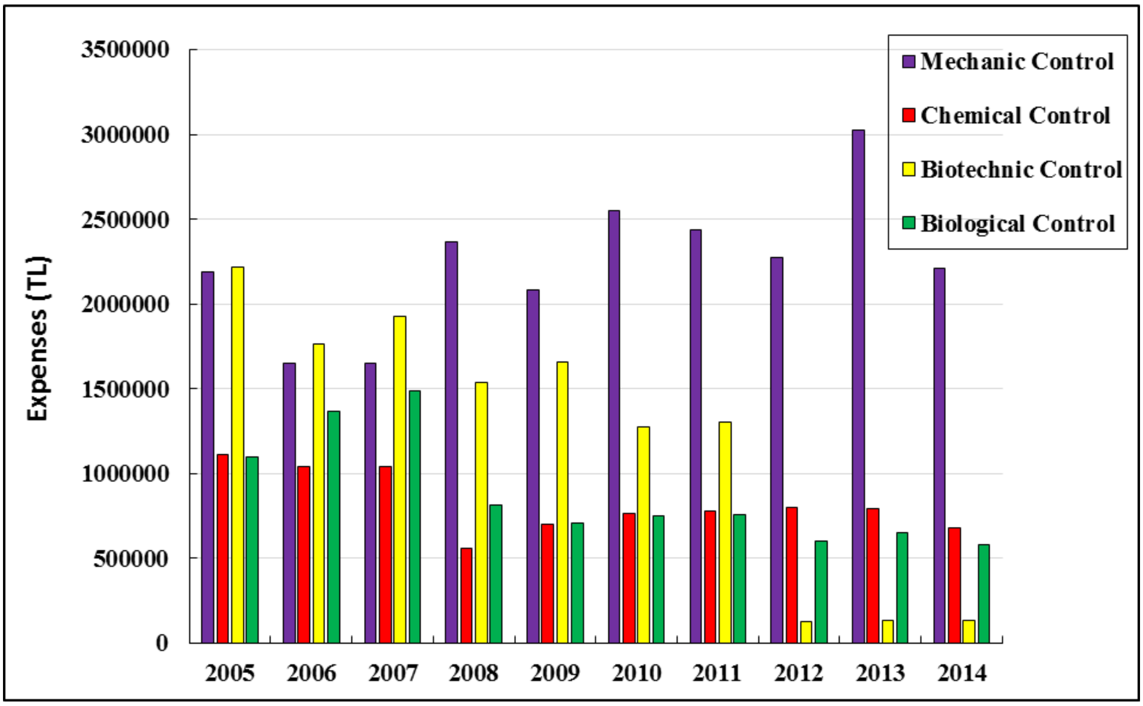

Figure 7. *Distribution of the costs of control methods used in Turkish forests (2005-2014)*

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This is followed by biotechnic control methods. *Fig. 7* shows that the expenditures have declined in recent years thanks to the success of biotechnical control. As for chemical control, the expenditures of GDF fluctuated from 2005 to 2014; overall it had a declining trend. However, as shown by *Table 1*, although the surface area where the GDF applied chemical control decreased from around 150 thousand ha to 10 thousand ha, it is noteworthy that the expenditures incurred for chemical control did not decline at the same rate. On the other hand, the cost of chemical control declined significantly from 1.113.000 TRY in 2005 to 680.125 TRY in 2014 (*Table 1*). In other words, although the area where chemical control was applied decreased by 15 times, the money spent for it decreased by only 1.6 times. One of the most important factors leading to that result was that almost all pesticides used for chemical control were imported and the TRY-USD exchange rate was high. Another factor is the need to reapply pesticides in the areas where chemical control failed.

Table 1. *Area-cost chart for biological and chemical control practices (2005-2014)*

Years	Chemical control			Biological control		
	Areas (ha)	Control costs (TRY)	Adjusted to 2015 (TRY)*	Areas (ha)	Control costs (TRY)	Adjusted to 2015 (TRY)*
2005	156.103	1.113.000	2.290.658,05	133.430	1.100.000	2.263.902,83
2006	170.379	1.038.499	1.915.544,61	120.153	1.366.706	2.520.932,92
2007	174.741	1.039.411	1.809.675,40	130.099	1.489.248	2.592.867.95
2008	102.415	557.000	897.047,74	128.475	814.000	1.310.945,90
2009	113.550	700.195	1.064.523,23	144.109	709.527	1.078.710,89
2010	76.184	763.515	1.066.188,16	178.897	752.145	1.050.310,86
2011	59.681	780.326	961.530,45	163.279	760.145	936.663,09
2012	39.741	800.269	962.497,97	108.641	602.589	724.744,67
2013	17.257	790.562	888.856,37	125.670	651.235	732.206,18
2014	5.729	680.125	718.970,33	120.751	576.956	609.908,84
Total	**915.780**	**8.262.902**	**12.575.492,31**	**1.353.504**	**8.822.551**	**13.821.194,13**

**Domestic Producer Price Index (D-PPI) was used for conversion (TUIK, 2017).*

Regarding biological control, the expenditures increased from 2005 to 2007, while decreasing in subsequent years (*Fig. 7*). This is because the GDF increased biological control practices by increasing the rearing of *C. sycophanta* in 2005, *T. formicarius* in 2006, and *R. depressus* in 2007. The decrease in the cost of biological control starting from 2008 was associated with the effectiveness and success of the control efforts (*Table 1*). Another point is that the average size of the areas where biological control methods were applied also varied significantly over the years. The average size of these areas decreased over the past years, while the reduction in the costs was more remarkable. This shows that the cost of biological control decreased as the natural balance was restored over the years (*Table 1*). As *Table 1* indicates, the costs in different years were converted to 2015 values for a more robust and accurate interpretation of the total costs of biological and chemical control over a period of years. At that stage, the unit costs were also calculated in order to determine the estimated amount of savings when biological control was preferred over chemical control (*Table 2*). To calculate the estimated amount of savings, it was assumed that biological control

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was preferred to chemical control. Thus, it was presumed that chemical control method was abandoned in the areas where biological control was applied, and the opportunity cost derived was retained by the GDF as savings. Accordingly, the estimated amount of savings in 2015 when the GDF preferred biological control to chemical control was around 1.75 million USD. Furthermore, in addition to this economic profit, the GDF also improved the effectiveness and success of control efforts from 2005 to 2014 on a continuous basis.

Table 2. *Unit costs of chemical and biological control and total savings*

Control Method	**Total Control Cost (TCC)** *TRY*	**Total Area (TA)** *ha*	**Control Unit Cost[1] (CUC)** *TRY/ha*	**Control Unit Cost[*] (CUC)** *USD/ha*	**Total Savings[2] (TS)** *USD*
Chemical Control	12.575.492,31	915.780	13,73	5,05	
Biological Control	13.821.194,13	1.353.504	10,21	3,75	1.751.840,23

*[1]CUC = TCC / TA, [2]TS = TBCA * (CCUC-BCUC)*
TBCA: Total Biological Control Area, CCUC: Chemical Control Unit Cost, BCUC: Biological Control Unit Cost
[*]Central Bank of Turkey average TRY-USD exchange rate of 2015 (1 USD = 2.72 TRY)

The economic analyses on biological and chemical control show that the cost of biological control is 3.75 USD per ha, compared to about 5.05 USD per ha for chemical control. These findings indicate that chemical control is 1.4 times more expensive than biological control in Turkey. An evaluation reveals that one of the most important factors affecting this difference is that the pesticides used for chemical control are imported and expensive. Moreover, harmful insects became resistant to the insecticides, requiring supply of new chemical compounds. This factor increases the cost of every new insecticide. For chemical control to succeed, pesticides have to be applied to the area until results are seen, which is another reason for the increased cost. Furthermore, special protective clothing, equipment and tools are needed to apply the pesticides in the field during chemical control. Annual periodical maintenance also increases the cost of chemical control. The cost of biological control is high initially, but decreases as the balance of the ecosystem is restored over time. The predators left in the area reproduce on their own and reduce the pest insect population below the economic loss threshold. Typically, it is not necessary to transplant the predators to the site every year. In other words, repetition is either not needed or very limited, which decreases the overall cost significantly. Another point related to cost is that the GDF employs its own laboratories and technical staff for the rearing of predators. Contrary to chemical control, they are not imported; therefore, the cost of rearing predators is low. Moreover, there is no need to use special clothing and equipment during the field application of biological control, which also decreases the cost.

Conclusions

In this study, the current status of biological control practices addressing pest insects in Turkish forests was analysed and compared with chemical control methods from an economic perspective. The first biological control was applied in Turkish forests in 1967, when *Formica rufa* was transplanted. However, the efforts regarding biological

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control were not that extensive until the 2000s. Turkish forestry organizations needed to take action because: the pesticides used with chemical control were harmful for the forests and human health, the chemical compounds used for the preparation of these pesticides were expensive, and the insects developed resistance against the pesticides. As a result, biological control efforts were increased especially after 2000 in Turkish forests, while the chemical control practices were eventually abandoned. This indicates the importance that the Turkish forestry organization attaches to the biological control method especially in recent years.

In particular, *C. sycophanta* since 2004, *T. formicarius* since 2006 and *R. depressus* since 2007 have been predominantly reared and extensively used during biological control operations. The quantity of these species and the areas where they are used have increased over the years and significant achievements were obtained. It is important from the ecological perspective that natural balance is restored gradually in these areas where biological control is applied. However, the economic aspects of insect control efforts are as important as the ecological aspects. From this perspective, an important finding of this study was that significant economic savings were obtained in addition to the ecological benefits as the chemical control was abandoned. The successful results including cost savings obtained by biological control practices add to point to the fact that the ecological balance in Turkish forests is gradually being restored.

The economic analyses revealed that the cost of biological control was around 3.75 USD per ha as compared to around 5.05 USD per ha for chemical control. Thus, when the ratio of the unit costs are calculated, it was found that the chemical control method was around 1.4 times more expensive than the biological control method. It is estimated that the economic savings will further increase as the areas where biological control is applied are extended, and the areas where chemical control is applied are reduced. Indeed, as the ecosystem gains back its natural balance in time through biological control, the cost of biological control per unit area will decrease; therefore, it is estimated that the economic savings will further increase. Thus, biological control is the most economic method for Turkish forests. The analyses revealed that the economic saving derived by abandoning chemical control methods is estimated at around 1.75 million USD in 2004-2014.

In conclusion, the primary benefit of biological control is that it restores ecological balance and thus ensures continuity of the ecosystem services. Its secondary benefit is the cost savings it brings. Therefore, the use of biological control methods to address pest insects in Turkey's forest ecosystems contributes remarkably to ecological and economic sustainability.

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EFFECTS OF PRE-GERMINATION TREATMENTS, SALT AND WATER STRESSES ON GERMINATION OF *ACACIA EHRENBERGIANA* HAYNE AND *ACACIA SEYAL* DEL. (MIMOSOIDEAE): TWO ALGERIAN NATIVE SPECIES

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Abstract. *Acacia ehrenbergiana* Hayne and *Acacia seyal* Del. are two desert trees of the Algerian Sahara that for a long time were confused. These two species present high potentials for desert areas rehabilitation therefore for successful seed germination and establishment three pre-germination treatments (manual scarification, boiling water and concentrated sulphuric acid) were used to overcome seeds dormancy. Once the optimal conditions are determined, we examined the effects of two abiotic stresses (salinity and water deficiency) on their seeds germination using eight levels of salinity (0, 50, 100, 150, 200, 250, 300 and 350mM of NaCl) and six concentrations of PEG_{6000} (0, -2, -4, -6, -8 and -10 bars) on *Acacia ehrenbergiana* Hayne and *Acacia seyal* Del. Germination rate and mean germination time were calculated and data were subjected to analysis of variance (ANOVA) followed by Newman-Keuls test. Our results show that manual scarification and sulphuric acid had significant effects on the seeds germination of *Acacia ehrenbergiana* Hayne meanwhile, only sulphuric acid enhanced *Acacia seyal* Del. seeds germination. Under salt and water stresses, the germination rate and mean germination time of *Acacia ehrenbergiana* Hayne were less affected than those of *Acacia seyal* Del. Therefore manual scarification and sulphuric acid treatments could be applied and adopted at laboratory level or even at nurseries to raise plants germination and plantations establishment for desert areas rehabilitation. Thus, *Acacia ehrenbergiana* Hayne seems to be more suitable for massive propagation as it demonstrates resilience when facing harsh abiotic stresses (salt and water stresses).
Keywords: *Acacia trees, seeds, germination, coat dormancy, abiotic stress, desert*

Introduction

Native acacia trees are frequent, widespread and emblematic species that grow in Algerian desert areas which cover 2/3 of the country surface. They are represented by six species; five of them were recently transferred from the genus *Acacia* to the genus *Vachellia*: *Acacia tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi) Brenan., *Acacia nilotica* subsp. *adstringens* (Schumach. & Thonn.) Roberty, *Acacia nilotica* subsp. *tomentosa* (Benth.) Brenan, *Acacia ehrenbergiana* Hayne, *Acacia seyal* Del. and one species: *Acacia laeta* R.Br. ex Benth. was transferred to the genus *Senegalia* (Ross, 1979; Kyalangalilwa, 2013).

These species play fundamental roles in desert areas, allowing sand's fixation, helping the water infiltration and improving the redistribution of nutritive elements in the soil. Moreover, their ability to develop dual symbiosis with *Rhizobia* and endomycorrhizal fungi contribute to the improvement of soil fertility (Grouzis and Le Floc'h, 2003; Noureddine et al., 2010). These prominent, wide range ecological services

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providers are well adapted to the main constraints of arid and semi-arid areas (drought, high temperatures, salinity and extreme light…) therefore, they are valuable trees and good candidates to combat desertification and to integrate any restoration, rehabilitation and reforestation programs in Algeria. Indeed, they have been already included as elite species in the Great Green Wall project, a project of plantation initiated by sahelian countries to fight the desertification progress (Dia and Niang, 2010; Ba, 2010; Hannani and Chehma, 2012).

The propagation and the distribution of these species require higher germination and establishment rates. One of the obstacles of acacias seed germination is the impermeable teguments to water and oxygen causing a physical dormancy (Holmes et al., 1987). In this case, it is necessary to apply pre-germination treatments to overcome the coat dormancy. Several artificial methods have been used to break the dormancy of these seeds, including physical (manual scarification, boiling water and cold water) and chemical pre-germination treatments (sulphuric acid and organic solvents) (Hanna, 1984; Cavanagh, 1987; Côme, 1970; Mousavi et al., 2011; Aref et al., 2011).

In addition, studies of the effects of most frequent environmental stresses encountered in these areas such as salt and water stresses on germination stage are of outmost importance for regeneration and consequently successful establishment of plantations (Aref et al., 2004; Jaouadi et al., 2010).

Studies have been carried out in order to determine the effects of pre-germination treatments, salt and water stresses on acacias seed germination (Teketay, 1996; Al-Mudaris et al., 1999; Ndour et Danthu, 1999; Jaouadi et al., 2004; Aref et al., 2004; Jaouadi et al., 2010; Kassa et al., 2010; Abari et al., 2011).

In Algeria, few published articles focused on these species including the works of Kebbas et al. (2015) and Hannani et al. (2016) on the effects of pre-germination treatments on *A. tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi) Brenan. and the studies of Karoune et al. (2013) and Kebbas et al. (2013) on the characteristics of germination under salt stress of *A. albida* (Del.) A. Chev. and water stress on *A. tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi) Brenan. respectively. Recently, Kheloufi et al., 2016 investigated the germination behaviour and compare the levels of tolerance to salinity in three *Acacia* species (*Acacia delbata* Link., *Acacia ehrenbergiana* Hayne and *Acacia tortilis* (Forssk.) Hayne var. *raddiana.*

A. ehrenbergiana Hayne which is found in the North Sahel, the Southern and Central Sahara, the Arabian Peninsula and East Africa resembles the *A. seyal* Del. Sudano-Sahelian species (Benchelah et al., 2006; Contu, 2012). *A. ehrenbergiana* Hayne grows in dry semi-desert areas where it represents one of the most drought-tolerant species among the common African acacias occurring in the rainfall belts 50-400 mm. In Algeria, this species can be found in Ahaggar, Tassili n'Ajjer and Tinghert Hamada while *A. seyal* Del. stands are rarely found in Ahaggar (Sahki and Sahki, 2004; Contu, 2012). Moreover, *A. ehrenbergiana* Hayne has not been mentioned in the flora dedicated to the Sahara; in Ozenda (1958) and Quezel and Santa (1962-1963); a raison of its confusion with *A. seyal* Del. (Celles and Maniere, 1980; Sahki and Sahki, 2004; Benchelah et al., 2006).

So, this study aims to describe seeds morphological characteristics of *A. ehrenbergiana* Hayne and *A. seyal* Del., two native species of the south of Algeria (Tamanrasset). Furthermore, we determined the effects of pre-germination treatments, salt and water stresses on their germination.

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Material and methods

Seeds of *A. ehrenbergiana* Hayne and *A. seyal* Del. were collected from mature pods of healthy trees in 2013 from Tamanrasset (22° 47' 13" North and 5° 31' 38" East) which is located in the south part of Algeria, about 2000 km far from the capital Algiers.
Seeds of *A. ehrenbergiana* Hayne were collected from Amlawlawen area about 15 Km far from Tamanrasset city while *A. seyal* Del. seeds were collected from Taghemout area approximately 50 km far from Tamanrasset city.

In the laboratory of soil biology in 2014, the seeds were removed from their pods, damaged and insect infected seeds were discarded and the empty ones were eliminated using the floating method (Ahmadloo et al., 2011). The seeds were stored at laboratory conditions at 2-5 °C and 80% of humidity.

Seeds were evaluated with both a light microscope (ZEISS Primo Star) and a scanning electron microscope (Jeol JSM 6360LV). Their morphological characteristics: colour, shape, texture, areole shape and position, shape of the hilo-micropylar region, spermoderm brightness, seed weight refers the weight of 1,000 seeds according to ISTA (the average of 10 replicates of 100 seeds weight multiply by 10), size, volume, surface, viability and water content were evaluated (Baldwin, 1942; Justice, 1972; ISTA, 1993).

Effects of pre-germination treatments on acacias seeds germination

Seeds dormancy of acacias species is associated with seed coat impermeability to water and oxygen which constitutes in one hand an adaptive factor for their survival but in the other hand, it is an obstacle for germination even when conditions are optimal. So, in order to overcome the seeds dormancy, we performed three pre-germinations treatment, we applied Manual Scarification (MS) which consists of careful nicking or filing seed coat by hand with a nail clipper on the opposite side of the seed embryo to allow water to inter into seeds. We used Boiling Water (BW) which involves soaking the seeds in boiling water for one hour in order to soften the seed coat. And finally, soaking seeds in concentrated Sulphuric Acid (SA) H_2SO_4 96% during one hour to weaken seed coat is conducted (Danthu et al., 1992; Ndour, 1997).
In order to minimize any fungal infection of the seeds during the incubation period, seeds were washed with distilled water then their surface were sterilized by immersion in ethanol 70° for 30 seconds and in sodium hypochlorite solution 0.58% for one minute followed by three washes in sterilized distilled water. All these steps where done in a sterile hood.

Five replicates per treatment, with 20 seeds placed in plastic Petri dishes on filter paper were incubated in the darkness because of the seeds light insensitivity (Danthu et al., 2003) at 25°C (primarily experiments) and under a relative humidity of 80%.

Effects of salt and water stresses on acacias seeds germination

From the results of the pre-germination treatments, optimal conditions were determined and used for further experiments. After applying the sterilization and germination protocols, salt stress effects on seeds germination at different levels of NaCl (0, 50, 100, 150, 200, 250, 300 and 350 mM) were evaluated and different solutions of PEG_{6000} (0, -2, -4, -6, -8 and -10 bars) were used to induce osmotic stress (Michel and Kaufmann, 1973).

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The seeds were humidified with NaCl and PEG_{6000} solutions every 48 h under sterile conditions and counting the germinated seeds which was defined as the emergence of the radical was done.

Determination of germination parameter

The Germination Rate (GR) and Mean Germination Time (MGT) were determined according to Côme (1970).

$$GR = n/N * 100 \qquad \text{(Eq.1)}$$

Where N is the total number of seeds and n is the number of germinated seeds.

$$MGT = N_1T_1 + N_2T_2 + \ldots + N_iT_i / N_1 + N_2 + \ldots N_i \qquad \text{(Eq.2)}$$

Where N_1 is the number of germinated seeds in T_1 and N_2 the number of seeds that germinated between T_1 and T_2.

Statistical analysis

The data was represented as mean ± standard deviation (SD). The experiment was made as a completely randomized design with five replicates (n=5) of twenty seeds of each replicate. The data were statistically treated by one-way analysis of variance (ANOVA) and the mean values were compared using Newman-Keuls test at P< 0,05 to establish the significant differences between each treatment and control.

Results

Seeds characteristics of A. ehrenbergiana **Hayne** *and A. seyal* **Del.**

The morphological characteristics of the two acacias species studied represented in (*Table 1*) and (*Fig. 1*) show some similarities in seeds shape, texture, areole shape and position. *A. ehrenbergiana* Hayne have a light brown colour while *A. seyal* Del. have a greenish brown colour. Morphology of the seeds varied significantly (P < 0,05) in length, width, volume, surface and weight of 1000 seeds. The seeds showed a high viability of 90 and 95% for *A. ehrenbergiana* Hayne and *A. seyal* Del. respectively.

Effects of pre-germination treatments on acacias seeds germination

In this work, we investigated the effects of three pre-germination treatments to overcome the seeds dormancy of *A. ehrenbergiana* Hayne and *A. seyal* Del. All pre-germination treatments improved the GR and MGT for both species while the control group presented very low GR (13% for *A. ehrenbergiana* Hayne and 18% for *A. seyal* Del.) and we noticed an increase in the MGT of both species (7,79 days for *A. ehrenbergiana* Hayne and 6,78 days for *A. seyal* Del.).

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Table 1. *A. ehrenbergiana* Hayne *and A. seyal* Del. *seeds morphological characteristics*

Species	Seed colour	Seed shape	Seed texture	Areole shape	Areole position	shape of the hilo-micropylar region	Spermoderm brightness	Weight of 1000 seeds (g)	Seed size (mm)			Volume (mm^3)	Surface (mm^2)	Viability (%)	Water content (%)
									Length	Width	Thickness				
***A. ehrenbergiana* Hayne**	Light brown	Oblong	Smooth	Horseshoe	Subterminal	Round	Slightly shiny	23,26±0,99***	5,67±0,66***	2,58±0,50***	1,06±0,21	21,34±6,20***	36,86±7,18***	95± 0,81*	4,45±2,26
***A. seyal* Del.**	Greenish brown							36,98±0,92***	6,86±0,65***	3,72±0,66***	1,14±0,43	79,80±23,95***	88,82±17,19***	90±2,16*	5,37±1,75

Mean ± standard deviation, *** very highly significantly different (P< 0,001), * significantly different (P< 0,05) using the Newman-Keuls test

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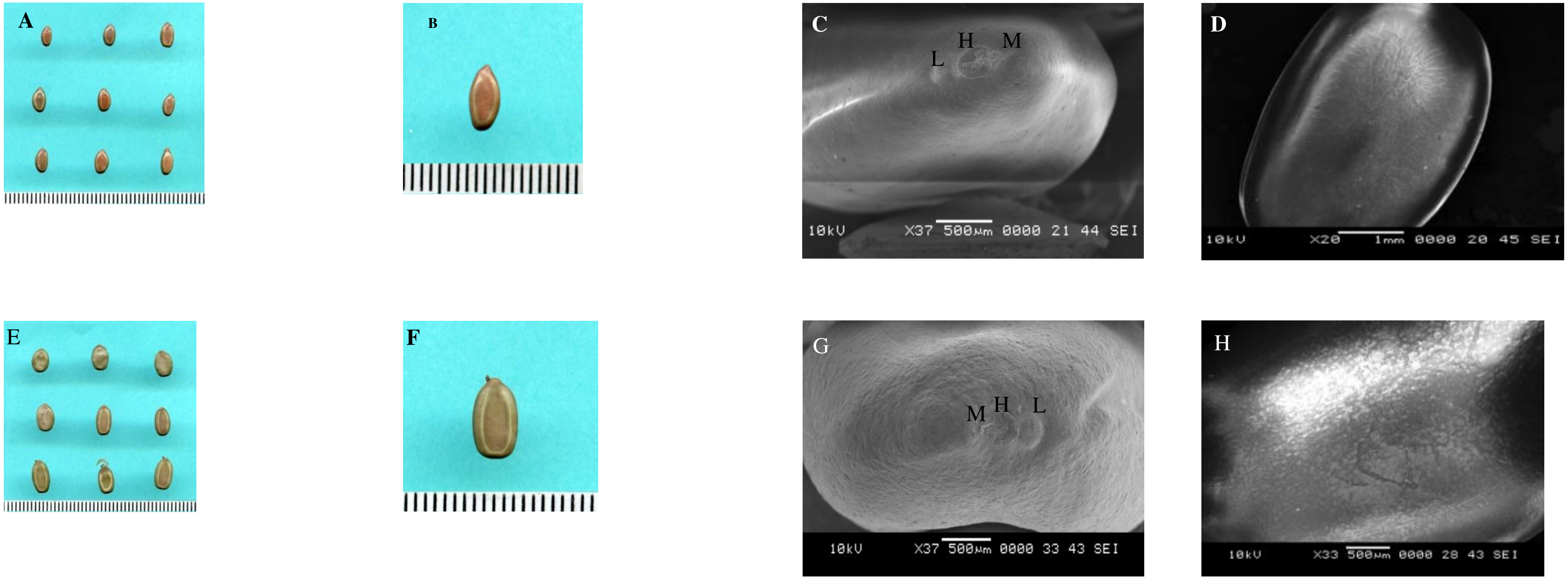

Figure 1. *Light microscope and scanning electron microscope study of the seeds morphological characteristics of A. ehrenbergiana* Hayne *(A, B, C and D) and A. seyal* Del. *(E, F, G and H), (**L**: Lens, **H**: Hilum and **M**: Micropyle)*

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Using sulphuric acid and manual scarification presented high significant difference (P<0,001) compared to the control group with the same and the best effects on *A. ehrenbergiana* Hayne seeds germination while sulphuric acid seems to be the best pre-germination treatment improving GR and MGT of *A. seyal* Del. Moreover, boiling water treatment came in the last position for both species giving a GR three times higher than the untreated seeds and two times lower than sulphuric acid and manual scarification (*Fig. 2*).

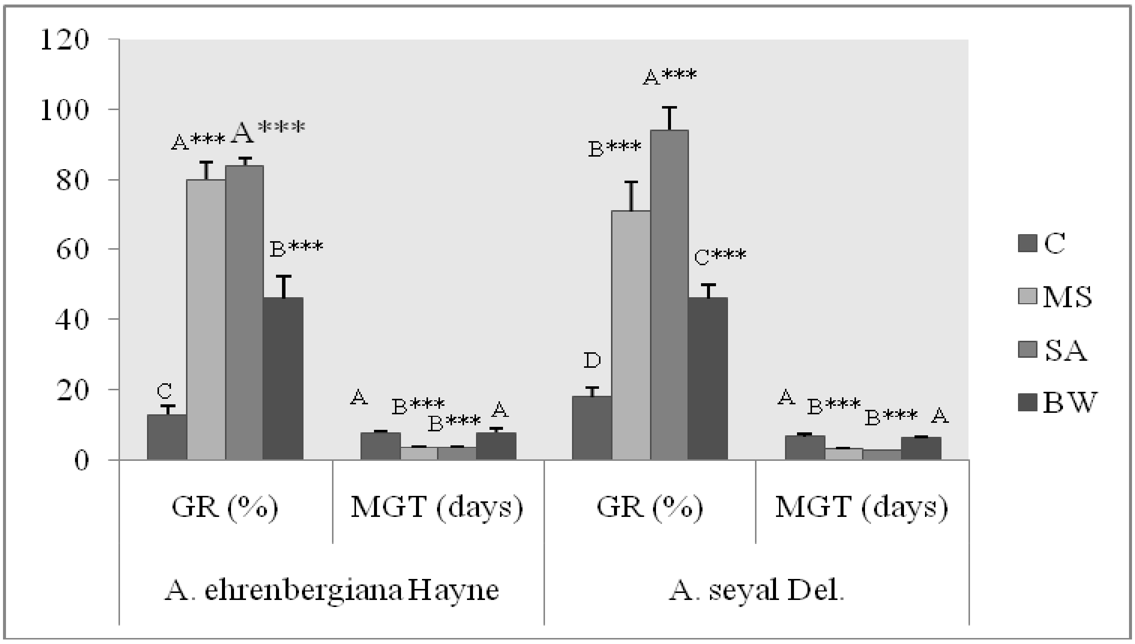

Figure 2. *Effects of manual scarification, sulphuric acid and boiling water on A. ehrenbergiana* Hayne *and A. seyal* Del. *seeds germination*

*Germination rate (GR), Mean Germination Time (MGT), Control (C), Mechanical Scarification (MS), Sulphuric Acid (SA), Boiling Water (BW), n=5, *** Very high significant difference with the control (P<0,001), A, B and C statistical similarity between treatments, Means followed by the same letter are not significantly different at P <0.05 using the Newman-Keuls test.*

Effects of salt and water stresses on acacias seeds germination

In our study, the germination behaviours under different environmental limiting conditions of salt and water stresses on germination of *A. ehrenbergiana* Hayne and *A. seyal* Del. were investigated.

Our results show that the highest GR and the shortest MGT occurred in the control group and we noticed an inverse correlation between GR and NaCl concentrations. Indeed, increasing salinity levels decreased GR and increased MGT presenting a very high significant difference (P<0,001) compared to control group for both species. Moreover, *A. seyal* Del. seems to be more sensitive than *A. ehrenbergiana* Hayne which germinated even at high levels of salt (at 350 mM) (*Table 2*).

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Table 2. *Effects of salt stress on GR and MGT of A. ehrenbergiana* Hayne *and A. seyal* Del. *seeds germination*

Species		***A. ehrenbergiana* Hayne**		***A. seyal* Del.**	
		GR (%)	**MGT (days)**	**GR (%)**	**MGT (days)**
C		77±2,73	4,7±0,84	82±2,73	4±0,48
Concentration of NaCl (mM)	**50**	63±2,73***	5,39±0,21	68±2,73***	5,28±0,50***
	100	52±2,73***	5,96±0,67**	58±2,73***	5,68±0,46***
	150	42±2,73***	8,47±0,55***	46±4,18***	6,99±0,28***
	200	30±3,53***	8,91±0,25***	33±4,47***	7,52±0,70***
	250	23±2,73***	9,47±1,18***	17±2,73***	9,28±0,29***
	300	16±2,23***	9,63±0,24***	12±2,73***	9,76±0,22***
	350	11±2,23***	10±0,00***	0±0,00***	0±0,00***

Mean ± standard deviation, Germination rate (GR), Mean Germination Time (MGT), Control (C), n=5, *** Very high significant difference with the control (P<0,001), ** high significant difference with the control (P<0,01) using the Newman-Keuls test

Germination assessment of *A. ehrenbergiana* Hayne and *A. seyal* Del. seeds under water stress was investigated using different concentrations of PEG $_{6000}$. The different water stress levels significantly affected their seeds germination. A reduction in GR and an increased MGT were observed with very high significant difference (P<0,001) when compared with the control group. Thus, seeds of *A. ehrenbergiana* Hayne seems to tolerate high levels of water stress (-10 bars) with a GR of 21% and an MGT of 8,92 days compared to *A. seyal* Del. which could not germinate at -10 bars (*Table 3*).

Table 3. *Effects of water stress on GR and MGT of A. ehrenbergiana* Hayne *and A. seyal* Del. *seeds germination*

Species	***A. ehrenbergiana* Hayne**		***A. seyal* Del.**	
Concentrations of PEG_{6000} (bars)	**GR (%)**	**MGT (days)**	**GR (%)**	**MGT (days)**
C	81±2,23	4,70±0,20	78±5,70	3,69±0,63
-2	71±2,23***	5,03±0,59	61±2,23***	5,47±0,42***
-4	60±3,53***	5,54±0,59	48±2,78***	6,12±0,67***
-6	53±2,73***	6,50±0,22***	36±2,23***	7,64±1,00***
-8	41±2,23***	7,49±0,62***	11±2,23***	9,33±0,62***
-10	21±2,23***	8,92±0,46***	0±0,00***	0±0,00***

Mean ± standard deviation, Germination rate (GR), Mean Germination Time (MGT), Control (C), n=5, *** very high significant difference comparing with control (P<0,001) using the Newman-Keuls test

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Discussion

Acacia ehrenbergiana Hayne and *Acacia seyal* Del. are two native species to Algeria that have diverse economic and ecological significance. However, their seeds dormancy that causes low germination percentage seems to be problematic for their usage in afforestation or restoration programs.

Seeds characteristics of A. ehrenbergiana **Hayne** *and A. seyal* **Del.**

The seeds of *A. ehrenbergiana* Hayne and *A. seyal* Del. that were examined by both a light microscope and a scanning electron microscope presented some morphological similarities in seeds shape and texture, areole shape and position, shape of the hilo-micropylar region and spermoderm brightness and some differences in colour, size, volume, surface and 1000 seeds weight.

We noticed that *A. ehrenbergiana* Hayne seeds are smaller than *A. seyal* Del. seeds while Al-Gohary and Mohamed (2007) found that seeds of *A. seyal* Del. were smaller than those of *A. ehrenbergiana* Hayne. Furthermore, in contrast of our study where we observed a light brown colour of *A. ehrenbergiana* Hayne seeds and a greenish brown colour of *A. seyal* Del. seeds, Waly (2012) reported the dark brown colour of *A. ehrenbergiana* Hayne while Al-Gohary and Mohamed (2007) reported the brown colour of *A. ehrenbergiana* Hayne and the yellowish brown of *A. seyal* Del. seeds

In our study, seeds of both species have an oblong shape while Al-Gohary and Mohamed (2007) mentioned an obovate shape for *A. seyal* Del. and an oblong shape for *A. ehrenbergiana* Hayne seeds. Due to environmental impact, genetic variations and strategies of adaptation to different environmental conditions and soil types, differences in colour and size were reported by different studies (Al-Gohary and Mohamed, 2007; Waly, 2012; Elmagboul et al., 2014).

The present investigation revealed a significant difference in 1000 seeds weight between the two studied species. In addition, similar results of 1000 seeds weight have been noticed when compared with standard Kew database for both species (26 g for *A. ehrenbergiana* Hayne and 42 g for *A. seyal* Del.).

The water content is a critical factor that influences seed life span and viability; thus, from the results of the water content of *A. ehrenbergiana* Hayne (4,45%) and *A. seyal* Del. (5,37%) we could classify these species as true orthodox seeds implying successful long term storage (Chin et al., 1989).

In the other hand, the viability of the two species was assessed by the tetrazolium test which consists on the measurement of how many seeds in a lot are alive and could develop into plants. This test show high percentages of 95% and 90% of viable seeds of *A. ehrenbergiana* Hayne and *A. seyal* Del. respectively indicating their good conservation. In contrast, the tetrazolium viability test conducted by Al-Hammad and Al-Ammari, 2017 revealed that most non-germinated seeds of *A. ehrenbergiana* (80%) were dead.

Effects of pre-germination treatments on acacias seeds germination

Acacia seeds have a coat dormancy that acts primarily as mechanical barrier limiting water and oxygen entrance. In natural conditions, hard seed coats are broken by fire, extreme temperatures, and digestive acids in animals stomachs or by the abrasion of blowing sand (Luna et al., 2009). In laboratory and nursery conditions, artificial methods are used. According to many authors, manual scarification, sulphuric acid, hot

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water and dry heat treatments can break down the coat inhibition (Sadhu and Kaul, 1989; Danthu et al., 1992; Al-Mudaris et al. 1999; Grouzis and Le floc'h, 2003; Abari et al., 2012; Karoune et al., 2013; Rasebeka et al., 2014).

In this study, we tested three methods to overcome seed dormancy in *A. ehrenbergiana* Hayne and *A. seyal* Del. We revealed that manual scarification and sulphuric acid treatments are the best pre-germination treatments for *A. ehrenbergiana* Hayne while sulphuric acid treatment enhanced the GR of *A. seyal* Del. In addition, boiling water presented low germination rates for both species.

The study of Teketay (1996) revealed that manual scarification had the best effect on *A. seyal* Del. seeds where the germination percentage reached 100%. In the other hand, Aref et al. (2011) indicated that manual scarification was the best pre-germination treatments for *A. ehrenbergiana* Hayne seeds where the germination percentage was 96 %, followed by sulphuric acid and boiling water with the germination percentages of 94% and 69 % respectively. In contrast of our findings, the work of Aref et al. (2000) show that boiling water had positive effect on *A. seyal* Del. germination percentage (34.25%) while it has less effect on *A. ehrenbergiana* Hayne germination with a germination percentage of 9.35%.

Effects of salt and water stresses on acacias seeds germination

Seeds germination is a complex process depending on the genetic and environmental factors. Abiotic factors, such as salt and water stresses limit plant germination and growth during early seedling stages (Mansour, 2000). Salt and water stresses affect plants in a similar way. The osmotic effect causes a deficient in water absorption that limits seed imbibition leading to a series of metabolic changes such as the reduction in seed reserves hydrolysis and their use. Salt and water stresses can completely inhibit germination and cause a reduction in plant growth (Ahmad and Bano, 1992).

The effect of salt stress on acacias seeds germination was described by several authors (Ndour and Danthu, 1999; Aref et al., 2004; Abari et al., 2011; Karoune et al., 2013) which showed that increasing salt concentrations decreased the GR of the studied acacias species. However, in all cases, the acacias species were considered as salt tolerant species and Marcar et al. (1991) indicated that acacias species differed in their salt tolerance.

Our results of germination behaviour of *A. ehrenbergiana* Hayne and *A. seyal* Del. show that both species germinated at low concentrations of NaCl (50 mM and 100 mM) with at least 50% GR, while the GR at high concentration (350mM) was decreased to 11% for *A. ehrenbergiana* Hayne seeds and it presented a total inhibition in *A. seyal* Del. seeds. This might be due to the inhibition of the radical emergence as a response to water deficit and their toxic effects on the embryo (Mazher et al., 2007). Jaouadi et al. (2010) suggested that *Acacia tortilis* (Forsk.) Hayne ssp. *raddiana* (Savi) Brenan. is significantly affected by 9 g L^{-1} of NaCl giving a germination rate of 60%, furthermore, this species continues to germinate even at high concentrations of NaCl reaching 18 g L^{-1} with a germination rate of 50%. In our case, *A. ehrenbergiana* Hayne could germinate even at high level of NaCl (350 mM = 17g L^{-1}) however it gave a GR of 11%. Also, the study conducted by Kheloufi et al. (2016) revealed that the seeds of *A. ehrenbergiana* Hayne collected in Oued Tin Amezzegin (Tamanrasset, Southern of Algeria) gave a final GR of 100% for the first three seawater treatments (0, 10, and 30 %) and 78 % in the presence of 50% of seawater.

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The results of the effect of water stress on *A. ehrenbergiana* Hayne and *A. seyal* Del. seeds showed that GR of both species were significantly affected particularly, *A. seyal* Del. seeds which presented an inhibition at high level of water stress (-10 Bars). Kassa et al. (2010), reported that this species can germinate even at high level of water stress (-12 MPa) and asserted that it is classified as tolerant species to water stress. In addition, Ndour and Danthu (1999) reported that *A. ehrenbergiana* Hayne is more tolerant than *A. seyal* Del. to water stress and both species are considered as tolerate species to water stress because they germinated even at -2,1 MPa.

Our research was lead by the lack of studies on the biology and characteristics of seeds of highly tolerant and beneficial trees in arid areas of Algeria. The present study show some important elements to distinguish between *A. ehrenbergiana* Hayne and *A. seyal* Del regarding their seeds morphological characteristics. In addition, because of their seed coat dormancy, we applied chemical and physical treatments in order to obtain rapid and synchronous germination essential for ensuring seedling establishment. We described the optimal germination conditions of these two species and established their germination behaviour under salt and water stresses two of the most frequent environmental stresses that prevail in arid areas of Algeria. These results are of a significant important as *A. ehrenbergiana* Hayne and *A. seyal* Del. two native species that present a good choice and could be suitable for land rehabilitation, restoration and afforestation programs in these areas.

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IMPACT OF FERTILIZERS ON SOIL PROPERTIES IN THE CASE OF *SOLANUM TUBEROSUM* L. DURING CONVERSION TO ORGANIC FARMING

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Abstract. In organic farming the use of mineral fertilizers is highly restricted; hence biofertilizers are often used. The aim of the research was to investigate the effects of different fertilizers on soil microbial communities and enzymatic activity after conventional production. A field with potato cultivation under conversion to an organic system was established. The nitrogen organic fertilizer Bioilsa and two microbial biofertilizers – UGmax and EM – were used. The highest values of dehydrogenase activity (DHA) were observed in the soil where UGmax was used alone. Application of biofertilizers also caused an increase of DHA in variants with Bioilsa fertilizer. The differences in activity of phosphatase in relation to application of different biofertilizers were observed, but it was higher than in variants with Bioilsa. Application of Bioilsa had a positive effect on activity of acid and alkaline phosphatases, but probiotic EM reduced this positive effect. The total number of bacteria and actinobacteria was the highest in EM variants, not fertilized with Bioilsa. In the case of the fungal population no correlation with fertilization was noted. We conclude that biofertilizers can activate the microbial and enzymatic activity of the soil after intensive agriculture production and can neutralize the negative impact on these parameters of nitrogen fertilizer.
Keywords: *biofertilizers, nitrogen organic fertilizer, soil microbiology, dehydrogenase activity, phosphomonoesterases*

Introduction

Organic farming avoids application of synthetic fertilizers and pesticides, uses organic inputs, promotes recycling for nutrient supply and emphasizes cropping system design and biological processes for pest management (Le Guillou and Scharpé, 2001; Araújo et al., 2009). In the European Union, organic production of agricultural products is certified and regulated by Council Regulation 834/2007 (2007). During conversion to organic production there are in force restrictions on the use of mineral fertilizers and plant protection products that are subsequently maintained. The soil fertility can be improved only by establishing a rotation with legumes or large amounts of manure. This paper hypothesized that biofertilizers are helpful to restore microbial balance in the soil after intensive agriculture production. The aim of the study was to investigate the

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impact of biofertilizers and nitrogen organic fertilizer on soil microbial communities and soil chemical properties in a farm under conversion to organic production.

Review of Literature

The soil microbial community plays an important role in maintaining soil fertility in an organic agriculture system. The microbial fertilization is beneficial especially for organic farming under the condition that microorganisms are not genetically modified. Biofertilization (e.g. using microbial fertilizers) is now a very important method of providing plants with their nutritional requirements without having an undesirable impact on the environment (Yassin, 2002; Abd El-Malek, 2005; Abou El-Yazied and Sellim, 2007). This method of fertilization is one of the many factors which play an important role in activating the enzymatic and microbial properties of the soil. It is particularly important in the system of organic farming because healthy soil is a crucial factor for both healthy plants and high yield.

In an organic system production of some crops is problematic in regard to nutrients, particularly enzymatic nitrogen. One of them is potato cultivation, the fourth most important food crop of the world. Potatoes are the most common crop in the world and are grown all over the world, production in Europe accounting for approximately 40% of world production. However, potato production is steadily decreasing. In Poland organic potato production covers 4% of the area of total organic farmland. The use of microbiological bio-agents (also referred as ‘biofertilizers’) can enhance tuber yielding and chemical composition of the soil; it has been studied for some years (Trawczyński and Bogdanowicz, 2007). Many different biofertilizers are available on the market for agricultural use. The products are claimed to enhance plant growth and yields and to improve soil fertility, but often their microbial composition is not specified in detail, making it difficult for the users to evaluate the product and for scientists to prove its effectiveness (Schenck zu Schweinsberg-Mickan and Müller, 2009).

One from many available biofertilizers is UGmax and probiotic Effective Microorganisms (EM). EM are microbial inoculants promoting stimulation of plant growth and soil fertility in agriculture. It is not clear whether EM contain microorganisms that can act as plant growth-promoting microorganisms (PGPM) (Meyer et al., 2010). UGmax is a microbiological preparation composed of yeasts, lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas* and *Actinobacteria*, as well as potassium. UGmax aims at improving physiochemical soil properties. It accelerates the decomposition of post-harvest residue and organic fertilizers, activates nutrients from minerals or insoluble compounds and improves water relations (Długosz et al., 2010). Thus, crops can use elements from mineral fertilizers more efficiently, which in turn allows farmers to lower mineral fertilization doses, consequently limiting the emission of harmful substances to surface waters. The positive effect on potato tuber yield volumes is also well known after biofertilizers (Zarzecka et al., 2011).

The use of microorganisms is a source of controversy. There is evidence in the literature of both their usefulness in agriculture (Sangakkara et al., 2011) and a lack of evidence (Mayer et al., 2008; Mayer et al., 2010; Martyniuk and Księżak, 2011; Martyniuk, 2011). These preparations contribute to the improvement of the biological activity of the soil, increasing the binding of free nitrogen from the air, reducing the erosion and loss of nutrients.

Unlike organic farming, conventional agriculture is largely dependent on intensive chemical inputs and has an important role in improving food productivity. However,

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organic farming is a sector which is able to secure the food needs for the growing number of consumers. It grew from $3.2 billion in 2008 to $5.5 billion in 2014, demonstrating increased demand for organic products and opportunities for growth (http://www.ofrf.org/organic-faqs). Many studies from around the world have compared organic and conventional farming systems in terms of soil properties. According to them microbial activity and nitrogen mineralization rates were higher under organic production than under conventional production (Workneh and van Bruggen, 1994). Enhanced soil fertility and higher biodiversity found in organic plots may render these systems less dependent on external input (Mäder et al., 2002). Organic farming with compost amendment showed the best results in terms of microbial biomass carbon, nitrogen and phosphorus (CNP) (Amaral and Abelho, 2016). Organic farming has been shown to favor soil biota in comparison with intensive farming (Kallenbach and Grandy, 2011; Santos et al., 2012). Field soils in organic farms were also more productive than conventional fields, probably due to the beneficial effects on soil properties of long-term organic amendments. The soil under the organic agricultural system presents higher microbial activity and biomass and lower bulk density than the conventional agricultural system (Araújo et al., 2009). In this context the changes taking place in the soil during the conversion period and after microbial fertilizers treatments applied to speed up the restoration to homeostasis are very interesting. The reality of contemporary agriculture necessitates the search for products improving the development and yield of crops, which are distinguished by a strong consequential impact in the entire crop rotation. When diagnosing the influence of different agrotechnical and chemical factors, including fertilizers, on the state of soil, enzymatic and microbial activity are considered to be good indicators of the soil environment quality because of their high sensitivity and rapid reaction. Monitoring the pedosphere by means of methods based on enzymatic tests enables complex assessment of changes taking place in the soil environment as a result of fertilization (Niewiadomska et al., 2010; Notrcliff, 2002; Tian et al., 2009).

Material and Methods

Study location and experimental designs

In 2014-2015 in the Experimental Station of the Institute of Plant Protection – NRI (western part of Poland, Wielkopolska region) in Winna Góra (52.2N; 17.4E) the experiment was established with potato cultivation under the first (2014) and the second year (2015) of conversion to an organic system (*Fig. 1*). On each experimental field wheat was cultivated as a forecrop. The experiment was carried out on plots (size plot 100 m^2 with randomized distribution) within the one big area. Due to the necessary rotation, experimental plots were changed in subsequent years, although they were located side by side and within the one big area under conversion to organic production. Type soil was classified as clay soil with classification III b. One potato cultivar was cultivated – Ditta. No manure fertilizer was used. During the experiments the weather conditions were noted (*Table 1*).

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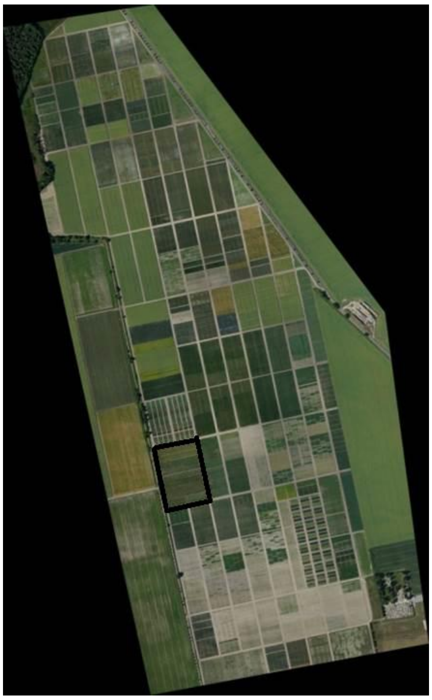

Figure 1. *Map of experimental site, the black line indicates the field during conversion to organic farming*

Table 1. *Mean monthly values of selected weather parameters*

	2014			2015		
	Temp (°C)	**RH (%)**	**precipitation (mm)**	**Temp (°C)**	**RH (%)**	**precipitation (mm)**
April	11.0	74	40.6	8.7	69	1.2
May	13.9	75	119.3	13.7	69	7.3
June	16.8	73	41.7	16.5	72	32.5
July	22.0	69	71.9	20.0	67	13.6
August	18.0	75	58.8	22.8	58	0.0
September	15.8	81	61.4	15.6	73	4.6

In the experiment organic nitrogen fertilization delivered by Bioilsa was used as the first research variant. The second research variant consisted of two biofertilizers, approved for organic plant production, probiotic EM (ProBiotics Polska) and UGmax (PPHU "Bogdan"), applied at a dose of 40 l and 1 l ha^{-1}, respectively. Effective microorganisms (EM) were a mixed culture of beneficial microorganisms including a predominant population of lactic acid bacteria (*Lactobacillus* sp.) and yeast (S*accharomyces* sp.), and a small proportion of photosynthetic bacteria (*Rhodopseudomonas* sp.), actinomycetes and fermenting fungi. No macro- or microelements are contained. UGmax contains lactic acid bacteria, photosynthetic bacteria, *Azotobacter*, *Pseudomonas*, yeasts, actinomycetes and some macro- and microelements (mg dm^{-3}), N–1200, P-220, K-2905, Mg-100, Na-200, Mn-0.3 (Sosnowski and Jankowski, 2013) These biofertilizers were applied in different methods, as soil and foliar application, due to the potential of biofertilizers to stimulate the natural defenses of potato plants. Before planting directly to the soil and during vegetation four foliar sprays, separately for each microbial product, were used at a 10-day interval. Thus, e.g. the first spraying was performed before row closure, and the third treatment was applied in full blossom. The experimental plots with biofertilizer treatments were divided into two separate parts in regard of Bioilsa application – applied or not. This fertilizer contains 12.5% organic nitrogen and 42% organic carbon, gradually releasing nitrogen and approved for organic farming (NPK 12:0:2). It was applied in the spring, 3 weeks before potato planting on selected plots at a dose of 300 kg/ha. This dose was chosen because it is mean between recommended doses, used as a standard in our tests and it was established on the basis of our experience. Three repetitions were made for each combination within one big experimental area and in each year.

Determination of carbon, nitrogen and phosphates in the soil

Soil samples for chemical analysis were collected at 30 cm depth (between rows of potatoes) from the experimental plots three times during each season, at the beginning of potato vegetation, at the beginning of plant drying and after harvest. The collected soil was dried in the open air and sieved through 2 mm-thick screens, then packed in a plastic bag. Unless specified otherwise, all reagents used in this study were analytical grade. The term “water” implies de-ionized water and was used for all sample extraction, soil sample preparation and standard solutions.

The standard phosphate solutions were prepared by measuring off the appropriate volume of the 100 $mg \cdot cm^{-3}$ o-PO_4^{3-}, prepared from KH_2PO_4 (Merck, Germany) standard solution into 100 cm^3 volumetric flasks and filling up to the mark with water. Serial dilutions were made to obtain the standard concentrations; 0.1; 0.2; 1.0; 2.0; 5.0; 10.0 and 20.0 $mg \cdot cm^{-3}$ phosphate-P. The extraction of phosphate-P was carried out according to the Egner-Riehm method. 0.04 M calcium lactate buffer (being acidified by hydrochloric acid up to pH 3.5 – 3.7) was used as an extracting agent. 200 cm^3 of the lactate buffer was added to the air-dried soil sample (approx. 5.000 g) placed in a screw-capped 500 cm^3 bottle. Activated carbon (approx. 0.20 g) was added and the bottle tightly screw-capped. The mixture was then shaken for 90 min on a Heidolph REAX 20-12 shaker (15 rpm). The phosphate-P in soil extract was measured photometrically via phosphomolybdate and reduction to molybdenum blue according to PN-EN ISO 15681-1:2006. The extracted samples were analyzed using a MLE FIA-System flow injection analyzer. All pH measurements were carried out using Elmetron CP-501 pH meter. The pH meter was standardized with use of standard buffers (Merck). A Heidolph shaker model REAX 20-12 was used for shaking all samples while extraction of phosphate-N was carried out.

In order to determine total content of nitrogen (N) and carbon (C) in the soil Vario MAX CNS Element Analyzer apparatus was used. The samples were dried at 105°C for 60 min and after that sample material was weighed in an amount of 1200 mg to reusable cups made of ceramic. The mineral soil program with max 4% content of C in a sample was used. Oxygen was dosed for 120 s in the amount of 100 ml/min with the threshold of O_2 15‰. Sandy soil was used as a certified reference material (Soil Standard Sandy OAS Cat No. B2180 – Batch no. 133506, Elemental Microanalysis Ltd) with content N=0.7 g kg^{-1} and C= 8.3 g kg^{-1}. Obtained results were N=0.68 g kg^{-1} with recovery 97.14%, and C=8.31 g kg^{-1} with recovery 100.12%. In determination of N and C we used the so-called daily factor for N=1.0036, for C=0.9884, which resulted from the signs of L-glutamic acid.

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Microbial analysis

In order to determine the microbial community total numbers of bacteria, actinobacteria and fungi were evaluated. The enzymatic soil activity was assessed by content of dehydrogenases, acid and alkaline phosphatase. Sampling was performed in this same way as for chemical analysis. After that each soil sample was transported to the laboratory in PCV bags, in the fridge but not frozen.

Enumeration of selected microorganisms

The plate method was applied to determine the populations of microorganisms in soil samples collected from under the plants, at a depth of 0-20 cm. Soil microorganisms were counted on adequate agar substrate (in five replications) and expressed in colony-forming units (cfu) g^{-1} dry weight (DW) of soil. The total number of bacteria was determined on Merck Standard agar after 72 hours (3 days) of incubation at 25°C; fungi were determined on a Martin (1950) medium after 5 days of incubation at 24°C; actinobacteria were assessed on a Pochon medium after 5-7 days of incubation at 25°C (Grabińska-Łoniewska, 1999).

Soil enzymatic activity

The analyses of the enzymatic activity of soil in different experimental variants were based on the colorimetric method applied to measure the dehydrogenase activity (DHA), where 1% TTC (triphenyl tetrazolium chloride) was used as a substrate. The measurement took place after 24-hour incubation at a temperature of 30ºC and a wavelength of 485 nm and it was expressed in μmol TPF $(24h)^{-1}$ g^{-1} DM of soil (Thalmann, 1968). The biochemical analyses of soil involved the determination of activities of acid (EC3.1.3.1) and alkaline (EC 3.1.3.2) phosphomonoesterases (PAC and PAL) by the method of Tabatabai and Bremner (1969). The activities were determined using as substrate disodium p-nitrophenyl phosphate tetrahydrate, after 1 hour incubation at 37°C at wavelength of 400 nm. Results were converted into μmol p-nitrophenol PNP h^{-1} g^{-1} DM of soil.

Statistics

The changes in the microbial composition of soil and enzymatic activity were analyzed statistically. Analyzed and computed data are the means of 3 samplings per year. The results were averaged because similar changes were observed after the use of the formulations throughout the experiment. The results (mean of 2014 and 2015) were subjected to analysis of variance by means of the Statistica 9.1 program. A one-way ANOVA was carried out to determine the effects of the cultivation methods. For the F-test showing significant differences, Tukey's test (HSD) at the probability level $p = 0.05$ was additionally performed to compare mean values.

Results

Dehydrogenases and phosphatases (acid and alkaline) are the most frequently investigated soil enzymes, because they exhibit noticeable reactions to stress factors (Bielińska and Mocek – Płóciniak, 2012). The analysis of variation in selected groups of soil microorganisms, such as total bacterial count, and the count of fungi and actinobacteria, is an adequate measure of soil bioactivity. The influence of different soil

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fertilizers on the microbiological state of soil was researched in the experiment. The following question arose: is the active ingredient included in the Bioilsa fertilizer strong enough to optimize the conditions of existence of bacteria improving soil properties and to acquire nutrients for agricultural production by potatoes, or is it better to apply the active ingredient with other commercially available biofertilizers, such as UGmax or soil probiotics in the form of EM. The univariate analysis of variance proved a highly significant influence (α=0.001) of the used fertilizers on the biochemical activity of soil under potato cultivation (*Table 2*).

Table 2. *F test statistics and significance levels of one-way analysis of variance for the activity of enzymes and number of selected groups of microorganisms associated with fertilizers regardless of type of trade product*

Soil parameter	F test statistic
Dehydrogenases	10.1619^{***}
Acid phosphatase	6.770^{***}
Alkaline phosphatase	3.8382^{***}
Total number bacteria	4.3473^{***}
Actinobacteria	4.5623^{***}
Fungi	2.1431^{ns}

Note: Standard deviation value was determined from five measurements.
**Statistically significant difference from control on significance level α = 0.05.*
***Statistically significant difference from control on significance level α = 0.01.*
****Statistically significant difference from control on significance level α = 0.001.*
DHA – dehydrogenases activity; PAC acid phosphatase activity; PAL - Alkaline phosphatase activity

The results show that the highest values of dehydrogenase activity (DHA) under the potato cultivation were observed in the variant with UGmax alone. They were much (157%) greater than in the control variant, where no fertilizer was applied (*Fig. 2*).

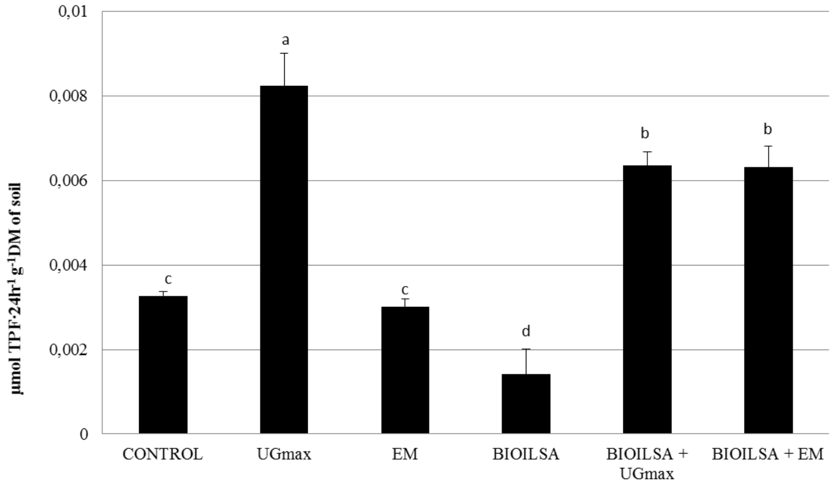

Figure 2. *The effect of different of fertilizers on the dehydrogenases activity*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a, b, c, d – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α=0.05; n=5

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The lowest dehydrogenase activity was observed in the variant with the Bioilsa fertilizer; it was 43% lower than in the control combination, where the activity of this enzyme was similar as in the combination with the probiotic formulation EM (*Fig. 2*).

The next indicator, acid and alkaline phosphatases activity, was statistically significantly higher (p=0.001) in the non-fertilized (control) variant compared with the other combinations. No differences in activity of phosphatase in relation to sole application of UGmax or probiotic EM were observed and it was higher than in variants with Bioilsa fertilizer. On the other hand, in the variant where Bioilsa and UGmax fertilizers had been applied simultaneously the lowest value of acid and alkaline phosphatase activity was observed (*Fig. 3* and *4*).

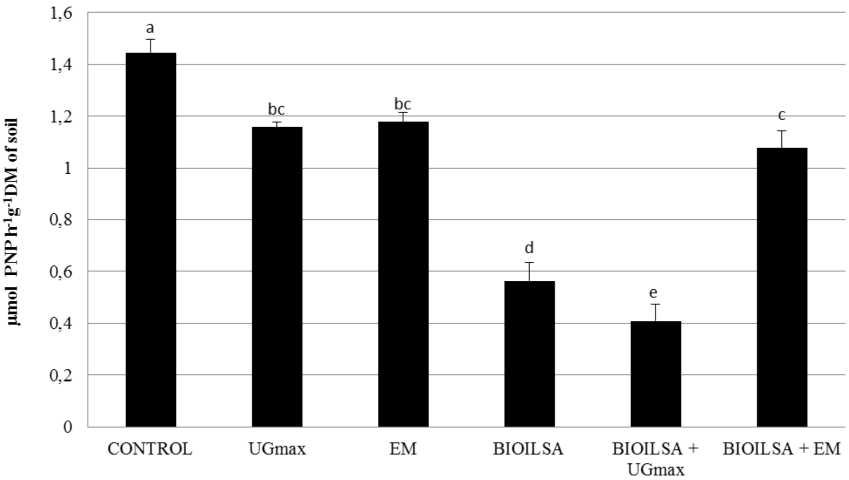

Figure 3. *The effect of different of fertilizers on the acid phosphatase activity*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a, b, c, d, e – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α=0.05; n=5

Generally, in variants with application of Bioilsa higher values of nitrogen, carbon and phosphorus were noted compared to variants without this organic fertilizer. The highest value (0.750 g kg^{-1}) of nitrogen was observed in variants with Bioilsa and without biofertilizers, but in combination without Bioilsa addition the biofertilizers can increase the content of nitrogen in the soil. However, it was not statistically confirmed (*Table 3*).

Table 3. *The content of the nitrogen, the carbon and phosphorus in the soil*

Plots with different type of fertilizers	**Total content/whole season, average value**		
	Nitrogen (N) g kg^{-1}	**Phosphorus P_2O_5 mg $(100g)^{-1}$ a.d.m**	**Carbon (C) kg^{-1}**
Bioilsa. non - biofertilizer	0.750	28	7.195
Bioilsa. with UG max	0.693	28.6	6.601
Bioilsa. with EM	0.696	45.7	6.550
Average for plots with Bioilsa	**0.721**	**34.1**	**6.782**
Non Bioilsa. with UGmax	0.721	30.8	6.703

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Non Bioilsa. with EM	0.713	28.2	6.593
Non Bioilsa. non – biofertlizer	0.705	25.5	6.770
Average for plots without Bioilsa	**0.713**	**28.16**	**6.688**

Note. a.d.m - air dried mass, – only indicative values are noted, with no statistical tests, samplings were taken three times within whole season

After application of the nitrogen organic fertilizer (Bioilsa) the average content of the nitrogen was higher (0.721 g kg^{-1}) compared to plots not fertilized with Bioilsa (0.713 g kg^{-1}); the content of the carbon was 6.78 g kg^{-1} in relation to plot not fertilized with Bioilsa (6.68 g kg^{-1}). The value of the last evaluated element, phosphorus, was 34.1 mg compared to non-fertilized 28.1 mg. Fertilization with Bioilsa in various modes influenced the parameters of chemical properties (*Table 3*).
The phosphorus content was higher in the soil after Bioilsa application; it may have caused that activity of phosphatases was lower compared to results obtained for samples collected from non-Bioilsa plots (*Fig. 3* and *4*). There were analogical observations about alkaline phosphatase (*Fig. 4*).

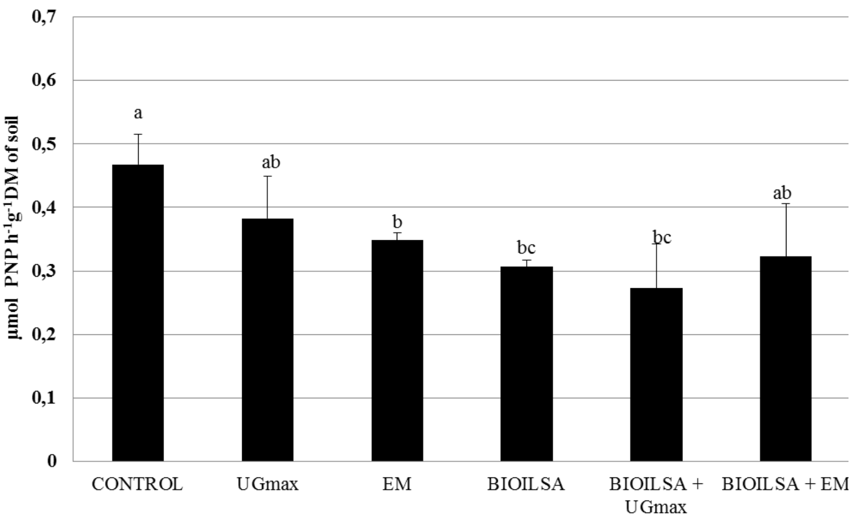

Figure 4. *The effect of different of fertilizers on the alkaline phosphatase activity*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a, b, c – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α=0.05; n=5

The results of the influence of all used fertilizers applied to the soil under potatoes on the number of selected groups of soil microorganisms point to a highly significant influence (α=0.001) on the changes in the number of total bacteria and actinobacteria in soil. This effect was confirmed by statistical tests (*Table 2*). This correlation was not observed in fungi (*Table 2*). The total number of bacteria and actinobacteria was the highest in EM variants not fertilized with Bioilsa. In variants with Bioilsa application the microbial products had an impact on increase of the population of these microorganisms (*Fig. 5* and *6*).

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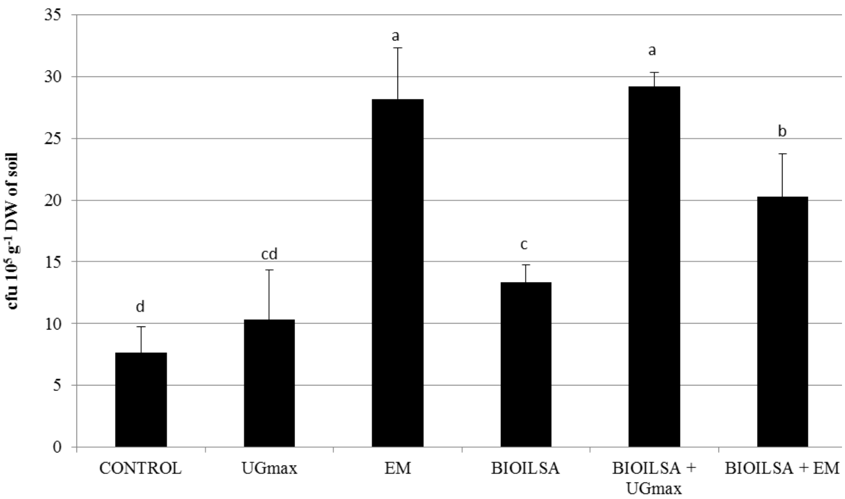

Figure 5. *The effect of different of fertilizers on the total number bacteria*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a, b, c, d – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α=0.05; n=5

According to Szember (2001), the latter group belongs to a so-called ecological group of microorganisms, which indicates soil fertility. It is noteworthy that the formulations applied to the soil contained certain amounts of macroelements (UGmax, Bioilsa), which may have increased the count of this group of microorganisms. There were similar results in the study by Swędrzyńska et al. (2011), who reported that there was a large increase in the count of actinobacteria when microbial formulations were applied to soil under perennial ryegrass. It is noteworthy that in our study the greatest increase in the count of these microorganisms was observed when probiotic EM had been applied (*Fig. 5* and *6*).

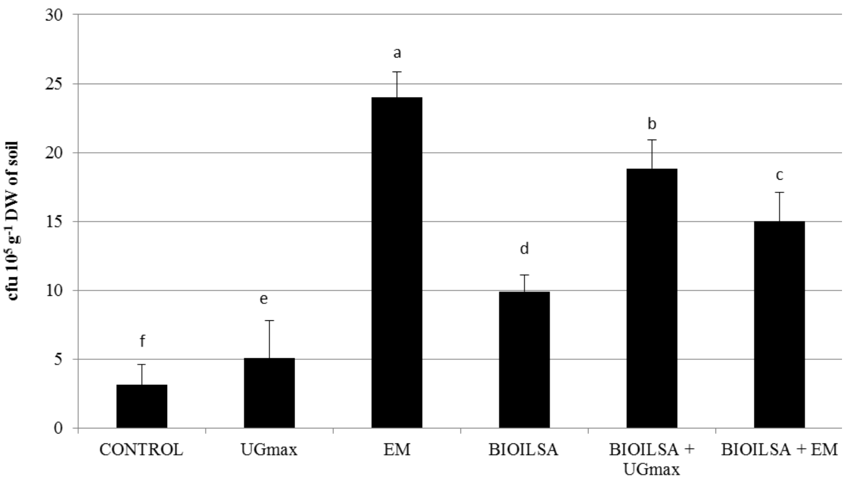

Figure 6. *The effect of different of fertilizers on the Actinobacteria*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a, b, c, d, e, f – homogenous groups according to Tuckey's test; different letters denote statistical differences at level α=0.05; n=5

The increase in the total count of bacteria and actinobacteria in these variants can be explained in two ways. The microorganisms supplied in the formulations may have been a perfect source of carbon for autochthonous bacteria colonizing the soil environment. On the other hand, the fertilizing formulation with macro-, microelements and microorganisms may have promoted the availability of nutrients and in consequence increased the count of these groups of microorganisms. There were similar correlations observed in fungi, but they were not statistically significant (*Fig. 7*).

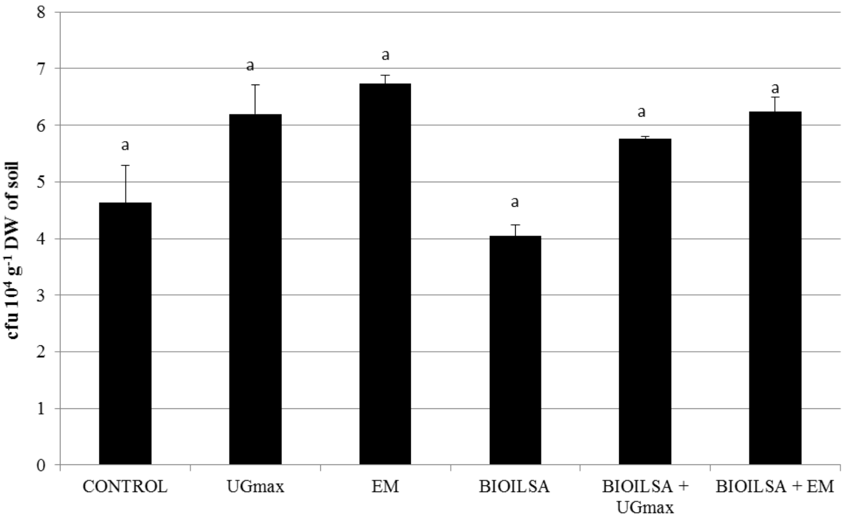

Figure 7. *The effect of different of fertilizers on molds (the fungi)*

Note: data are represented as means of five replications. Values after means are standard deviations ±; Means values ± standard errors; a – homogenous groups according to Tuckey's test; the same letter denote not statistical differences at level α=0.05; n=5

Discussion

According to Brzezińska and Włodarczyk (2005), DHA in the soil is an indicator of the intensity of respiratory metabolism of microorganisms, especially eubacteria and actinobacteria. The analysis of the activity of these enzymes provides information about the content of organic matter or fertility of the substrate. We can conclude that increased dehydrogenase activity in the variant where UGmax was applied to the soil was primarily caused by additional microorganisms included in this product as well as macro- and microelements contained in this fertilizer.

When measuring acid phosphatase activity it is necessary to remember that the amount of this enzyme in the soil environment is strongly influenced by plants, whose roots secrete considerable amounts of it, especially if they do not receive a sufficient supply of phosphorus (Burns, 1985; Dahm et al*.,* 1986; Żebrowska and Ciereszko, 2009). The high concentration of acid phosphatase in the control combination (where fertilization had not been applied) may have been the plants' response to a lesser supply of phosphorus in the soil environment. Many authors mention the fact that if there is a deficit of absorbable phosphorus in soil, plants' roots secrete higher amounts of phosphatase catalyzing hydrolysis of the C-O-P bond in organic phosphorus compounds (Schneider et al., 2001).

It is known that if phosphorus content is low, the activity of these enzymes is higher. According to Bielińska and Mocek-Płóciniak (2012), their long-term observations

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proved that the acid phosphatase enzyme was strictly correlated with the physiochemical properties of soil. We noted statistically significantly low activity of acid phosphatase in the variants where the nitrogen organic fertilizing formulation had been applied. This indicates that microbial formulations provide plants adequate amounts of phosphorus. Tarafdar and Rao (1990) reported a correlation between the uptake of phosphorus by plants, yield and acid phosphatase activity.

Microorganisms are attributed a significant role in the functioning of the soil ecosystem (Ahemad et al., 2009). They play a key role in controlling the reactions which are necessary to maintain soil fertility and structure. The soil is also characterized by bioactivity, which is indirectly influenced by soil microorganisms and the enzymes they secrete (Kieliszewska-Rokicka, 2001). The existence of soil microorganisms is not only limited to providing access to mineral compounds. They cause favorable transformations in soil, optimizing the living environment, growth and yield of plants. Positive effects on yield and plant healthiness after application of the same formulations were presented in the paper of Kowalska (2016). Apart from that, they participate in transformation of soil organic matter and are decisive to soil structure and its pH. They produce numerous bioactive substances in soil. Soil microorganisms detoxify many chemical compounds which are harmful to other organisms living in this environment, and they eliminate pathogenic organisms (Niewiadomska, 2013).

Conclusion

The research has shown that the highest values of dehydrogenase activity under the potato cultivation were observed in the variants with UGmax. Application of all tested fertilizers stimulated the availability of phosphorus to plants in the soil, because measurements showed the lowest phosphatase activity in soils collected from plots fertilized with Bioilsa. The positive effect of the different fertilizers UGmax and probiotic EM applied in the experiment on potatoes was also observed in the groups of soil microorganisms under study. Their number was greater than in the variant not fertilized with Bioilsa. When Bioilsa formulation was applied, the microbial products had a stimulating effect on the enzymatic activity or number of microorganisms. Such correlations were not observed only for the acid phosphatase activity after the application of UGmax. From the microbiological point of view the research findings should be regarded as promising. Our research indicated that biofertilizers can activate the microbial and enzymatic activity of the soil after intensive agriculture production and can neutralize the negative impact on these parameters of nitrogen fertilizer. We recommend continuing studies to confirm their effectiveness and usefulness in sustainable agricultural production.

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EFFECT OF FRAGMENTATION AND ANTHROPOGENIC DISTURBANCES ON FLORISTIC COMPOSITION AND STRUCTURE OF SUBTROPICAL BROAD LEAVED HUMID FOREST IN MEGHALAYA, NORTHEAST INDIA

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Abstract. The subtropical broadleaved humid forest of Meghalaya, northeast India are characterised by small remnant patches. The major threats to the existing patches are anthropogenic activities such as encroachment of forest area, mining, extraction of forest resources, grazing and forest fire. The present study was carried out at Jarain and adjoining areas of Jaintia Hills in Meghalaya, northeast India to identify the current human disturbances and assess the floristic composition and structure of subtropical broad leaved forest along a fragment size gradient. Floristic sampling was carried out by laying 24 plots (20mx100m) across 10 forest fragments covering a sampled area of 4.8 ha. A total of 160 woody species (≥5cm dbh) belonging to 105 genera and 54 families were enumerated from all the studied fragments. The species richness was 69 in Small, 75 in Medium, 76 in Very Large (VL) and 77 in Large (L) fragment classes. In this study, Pearson's correlation analysis was performed to analyse the relationship between area, disturbance and phytosociological attributes. The results showed that the stand density increased (r = 0.71, p=0.01), while basal area decreased with disturbance (r = -0.74, p= 0.01). The density was high in 5-15cm dbh class that gradually declined with the increase in diameter. The basal area was high in >66cm dbh class in Very Large fragments whereas in small patches, the values were higher in 16-25cm dbh classes. The forests fragments under study also have a number of rare, endemic and threatened species. Therefore, it is suggested that the entire landscape be brought under the protected area network due to high species diversity.
Keywords: *area, conservation*, *species diversity, density, Jarain*

Introduction

Fragmentation of large continuous tracts of forest into smaller patches leads to biodiversity loss, population shift, collapse of community assembly and ecosystem integrity (Mikkelson, 1993). The species richness in the fragments are determined by a number of attributes such as geographical location, topography, shape, management regimes, landuse history, internal disturbances (Ochoa- Guano et al., 2004) and the rate of colonisation and extinction of species over a period of time (Vellend et al., 2006). Though time scale is an indispensible variable in fragmentation studies particularly on plant community which are long lived and their response is slow, the debt of extinction due to fragmentation is inescapable (Vellend et al., 2006). There are two views regarding the effect of fragment size on biodiversity conservation. According to one of

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the views (the theory of island biogeography), fragments of larger area are supposedly assumed to harbour more species than the smaller fragments (Mac Arthur and Wilson, 1967). Larger fragments provide opportunity for establishment of varied microhabitats, space and resources to support more species and individuals (Schoener, 2010). Contrary to this, there are studies that have established that network of several small fragments have the potential of conserving biodiversity of a region (Toledo-Aceves et al., 2014; Ziter et al., 2013). In smaller fragments there is a high species turnover (Arroyo-Rodriguez et al. 2008). The consequence of fragmentation on species diversity may not be a linear species-area curve but are reflected through its response to edge effect which can cause cascading effects on the ecosystem. Tree species may be impoverished due to the loss of disturbance sensitive species in the fragments (Tabarrelli et al., 2004).

The larger fragments have more interior and are expected to support more of shade tolerant species (Benchimol and Peres, 2015). Species are susceptible to edge effect due to alteration in physical processes such as increase insolation, windthrow (Murcia, 1995; Laurance et al., 2000) and invasion by early successional species (Tabarelli et al., 2004). Gradual development of edge, affect the plant community since the ecological traits of the species may not be compatible with the conditions prevalent in the remnant habitat. The proportions of evergreen species in the edges are often replaced by deciduous elements associated with traits more adaptable to disturbance by shedding off their leaves to reduce water loss (Orihuela et al., 2015). Creation of edge may favour establishment of lianas (Laurance et al., 1998; Chettri, 2010) and light tolerant species (Ochoa-Gaona et al., 2004). However, epiphytes and herbaceous community may be threatened due to death of old growth trees that serves as host for the former and filter excess solar radiation to the herbaceous community (Hofmeister et al., 2013; Kolk and Naaf, 2015). Further, the invasion by light demanding species may pose competition with native old growth species that may alter the composition and structure of the community affecting ecosystem functions (Zhu et al., 2004).

Fragmented patches are more exposed to anthropogenic disturbances and small patches are highly susceptible to resource extraction that could further degrade the remnants (Cayuela et al., 2006). Although large reserves are usually preferred over smaller ones for conservation purpose, the effectiveness of forest patches to retain habitat core condition (Hofmeister et al., 2013), large subsets of species diversity and forest structure is still controversial (Lindenmayer et al., 2008; Gardner et al., 2009). There are studies that have established that species diversity increases with fragment size (Turner, 1996; Laurance et al., 2002). Contrary to this, species diversity has been observed to be independent of fragment sizes (Arroyo-Rodriguez et al., 2008; Hernandez-Reudas et al., 2014). Remnant patches can be of special interest irrespective of size because they may represent some of the only habitat left for certain species which are susceptible to disturbances particularly fragmentation. They may also have similar species diversity, complimentary to larger habitat necessary for biodiversity conservation and ecosystem services (Godefroid and Koedem, 2003). The consequence of fragmentation is of serious concern especially for species that are endemic, threatened or having ecological and economic interest.

The state of Meghalaya in northeast India is a part of the Indo-Burma hotspot (Mittermeier et al., 2004) and is rich in biological wealth and endemism (Upadhaya et al., 2012). The state supports various forest types ranging from tropical- evergreen, -semi evergreen to subtropical- broad leaved and -pine. The subtropical broadleaved humid forest found in higher altitudes (≥ 900 m asl) of the state have been recognised

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for their high species diversity and endemism (Upadhaya et al., 2003; Pandey et al., 2005; Jamir et al., 2006; Upadhaya, 2015). However, they occur in the form of highly fragmented patches and are poorly represented in the protected area network of the state (Upadhaya et al., 2013). The remnant subtropical forests are exposed to various kinds of disturbances and most of them have been converted to Pine *(Pinus kesiya)* dominated forest (Sarma, 2005). Other human activities such as overexploitation, deforestation, shifting cultivation, mining and urbanization have further contributed to the loss of subtropical broad leaved forest. Therefore, the present study was carried out at Jarain and adjoining areas of Jaintia Hills in Meghalaya, northeast India to:

- identify the current human disturbances operating in the remnant patches
- assess the floristic composition and structure of the forests along a fragment size gradient.

Materials and Methods

Study Sites

For the present study, forest fragments of different sizes ranging from 3.79 to 105 ha were selected at Jarain (E 25°19'20.45" - 25°19'25.55" and N 92° 07'42.33" - 92° 09'23.09", altitudinal range 1000m-1200msl), in Jaintia Hills of Meghalaya, northeast India. These patches were located within 5 km radius and represents similar climatic and edaphic conditions (*Figure 1* and *2*). The vegetation in the study area represents subtropical broad leaved humid forest (Champion and Seth, 1968). It is dense with short stature and the height of the tree rarely exceeds 18 m. The trees are distributed in three distinct strata and species like *Castanopsis* spp., *Daphniphyllum himalayenses, Eleocarpus lanceifolius, Engelhardtia spicata, Lithocarpus fenestratus, Magnolia insignis, Myrica esculenta, Persea odoratissima, Quercus semiserrata, Sarcosperma griffithii* and *Schima wallichii* usually forms the canopy layer. The species such as *Litsea elongata, Rhus acuminata, Syzygium tetragonum, Helecia nilagirica, Ligustrum robustrum* and *Lithocarpus dealbatus* forms the subcanopy layer. The undercanopy layer is dominated by large shrub or small trees such as *Camellia caduca, C. caudata, Daphne involucrata, Erythroxylon kunthianum, Eurya acuminata, Myrsine semiserrata, Symplocos spicata, Syzygium cuminii* etc. (Jamir et al., 2006; Pao et al., 2016).

The climate of the area is monsoonic with a distinct wet and dry period. The wet period extends from April to October during which more than 80% of the total rainfall occurs. The dry period extends from November to March with a rainfall of <20 mm and are characterized by low temperature (8ºC). In spring (April-May), the temperature starts to rise and there are few showers of rain as shown in *Figure 3* (Pao et al., 2016).

The Jaintia Hills is a continuation of the Meghalaya Plateau formed from the remnants of Pre-cambrian Indian peninsular shield. The rocks are mostly of Precambrian origin with gneissic composition such as granites, schist, amphibolits and calc-silicate (Central Groundwater Board, 2013). Soil texture of the studied sites varied from loamy sand to sandy. Bulk density ranged from 0.95 to 1.26 g cm^{-3} whereas, the porosity varied from 46.53 to 64.02%. The soil was acidic in nature (pH 5.4).

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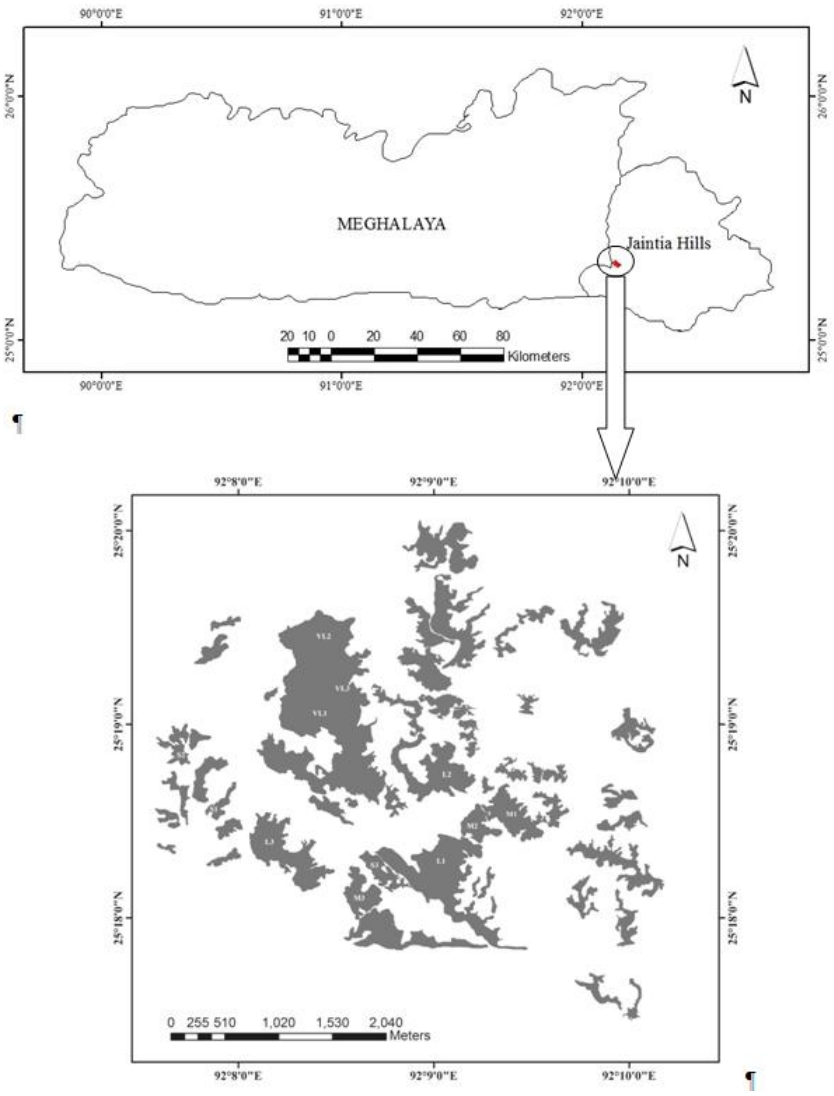

Figure 1. Map showing study area in Jaintia hills of Meghalaya, northeast India

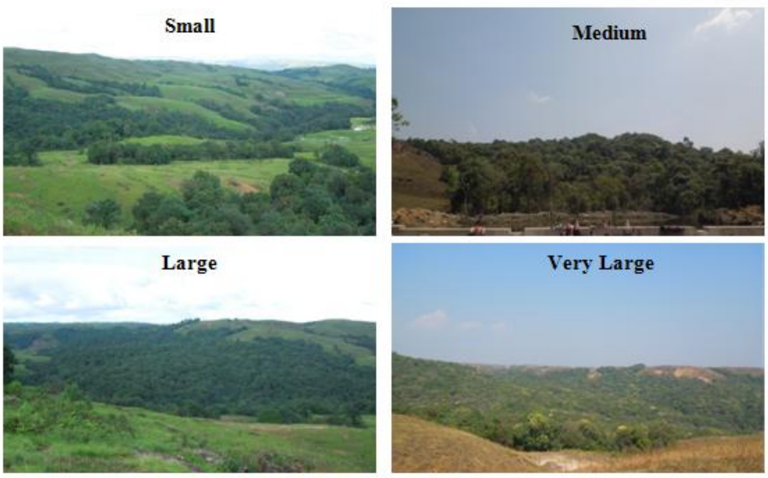

Figure 2. View of the landscape and some of the fragments in Jarain area of Jaintia Hills, Meghalaya, northeast India

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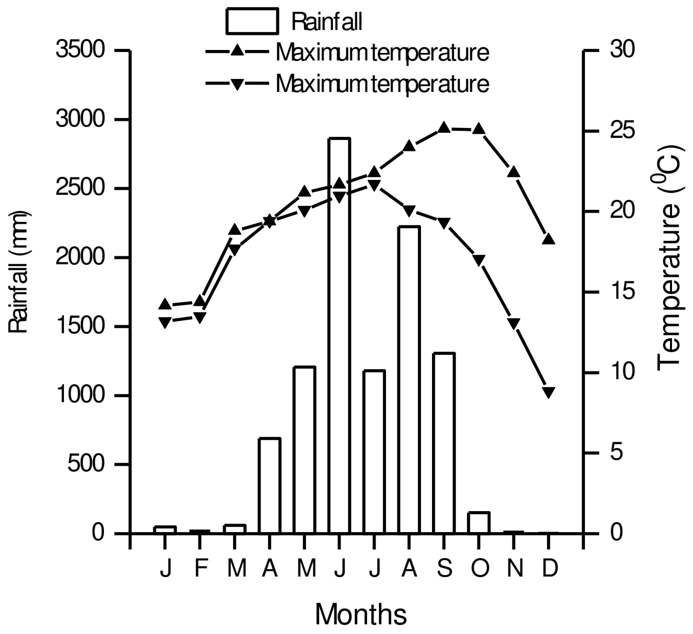

Figure 3. *Mean maximum and minimum temperatures and precipitation at Jowai for the year 2015 (Source:http://www.megagriculture.gov.in)*

Sampling Design

The 10 fragments of different sizes selected for the study were arbitrarily classified into four classes viz. Small (< 4 ha), Medium (>5and <15 ha), Large (>15 and <50 ha) and Very Large (105 ha) to study the phytosociological attributes along a fragment size gradient. Three replicate fragments were maintained for each fragment class. However, in the case of the Very Large, three replicated plots were maintained from a single large fragment due to lack of large continuous forests in the study area (Santos et al., 2007). These replicated sites were abbreviated as S (S1, S2, S3), M (M1, M2, M3), L (L1, L2, L3) and VL (VL1, VL2, VL3) respectively *(Table 1).* All the fragments were once a part of the same forest.

The disturbance index for each site was assessed following Mir et al. (2016). A score of 0 to 10 was assigned to each anthropogenic factor viz., extraction of -timber, -fuel wood, encroachment upon forest land for settlement, agriculture, mining and quarrying; road construction, NTFP's collection, construction of ponds, grazing and fire. A score of 0 was considered to be negligible, 1 as low, 5 as intermediate and 10 as high. The forest having all the disturbances at the highest intensity would have a total score of 110.

For vegetation sampling extensive field survey was carried out from 2013-2015. To capture the heterogeneity of structure and composition of the plant community, in each fragment, two transects of 20mx100m were laid, one along the edge of the forest while the other was laid at a distance of 50m from the first tree on the edge (Page et al., 2009). Each transect was further divided into 20 quadrats of 10mx10m size for inventorization of all woody species having a diameter at breast height (dbh) of ≥5cm. The specimens were collected and identified using regional floras and the Herbaria at Botanical Survey of India, Eastern Regional Circle, Shillong. The nomenclature of the species follows the regional flora.

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Table 1. *Neighbouring landscape unit and the current human disturbances in the study area*

Fragment Class	Site	Area (ha)	GPS coordinates		Neighbouring landscape unit	Current human disturbances	Disturbance
			Longitutdes	Latitudes			
Small	S1	3.79	N 25°19'25.55"	E 92°07'54.67"	Streams, road, grassland, reforested areas	Extraction of timber and fuel wood, fire, browsing, construction of -road, -pond, NTFP extraction	51
	S2	4.13	N 25°18'49.80"	E 92° 07'42.33"	Grassland	Extraction of timber and fuel wood, browsing, construction of road, NTFP extraction	35
	S3	4.99	N 25°18'15.63"	E 92° 08' 43.60"	Human settlement, road, grassland	Extraction of timber and fuel wood, fire, browsing, construction of road, encroachment due to human settlement, NTFP extraction	47
Medium	M1	13.2	N 25°18'37.22"	E 92° 09'23.09"	Pond, road, grassland	Extraction of timber and fuel wood, construction of - road, -pond, NTFP extraction	40
	M2	6.23	N 25°18'29.55"	E 92 °09'14.22"	Stream, grassland, road, stone quarry, crematory ground	Extraction of timber and fuel wood, browsing, construction of road, encroachment due to quarrying, NTFP extraction	27
	M3	8.72	N 25°18'10.70"	E 92 °08'37.97"	Human settlement, grassland, coal mine, settled agriculture, jhum plots	Extraction of timber and fuel wood, browsing, construction of road, encroachment due to -agriculture, -quarrying, -human settlement, NTFP extraction	70
Large	L1	28.7	N 25°18'08.15"	E 92°08'56.48"	Pond, road , stream, human settlement, cattle shed, settled agriculture	Extraction of timber and fuel wood, browsing, encroachment due to to -agriculture, -quarrying, -human settlement, NTFP extraction	61
	L2	19.6	N 25°18'40.39"	E 92°09'23.09"	Stream, human settlement, cattle shed, settled agriculture, ponds, hollow brick kiln	Extraction of timber, browsing, construction of pond, NTFP extraction	17
	L3	21.02	N 25°18'10.23"	E 92°08'25.19"	Ponds, road, coal mines, stone quarry, human settlement, farming	Extraction of timber and fuel wood, fire, browsing, construction of -road, -pond, encroachment due to -agriculture, -quarrying, -human settlement, NTFP extraction	39
Very Large	VL1	105	N 25°19'08.78"	E 92°08'39.41"	Stream, human settlement, grassland, coal mines, settled agriculture, jhum plots	Extraction of timber and fuel wood, browsing, encroachment due to mining, NTFP extraction	14
	VL2	105	N 25°19'20.45"	E 92°08'17.66"	Stream, coal mine, cattle raring, grassland, agriculture plots	Extraction of timber and fuel wood, fire, browsing, encroachment due to mining, NTFP extraction,	19
	VL3	105	N 25°18'56.73"	E 92°08'19.30"	Crematory ground, coal mines, quarry, grassland, roads	Extraction of timber and fuel wood, browsing, encroachment due to -agriculture, -mining, NTFP extraction	36

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Data Analysis

The vegetation data from the two transect for each sites were pooled together. Community parameters such as frequency, density, basal area and importance value index (IVI) were computed following Misra (1968) and Mueller-Dombois and Ellenberg (1974). Various diversity indices such as Shannon Wiener, Simpson and Pielou were calculated following Magurran (1998). Sorensen's index of similarity (Sorensen, 1948) was used for comparing the floristic similarity between the fragments. A check list of rare, endemic and threatened species occurring in the study area was prepared based on published literature (Pandey et al., 2005; Upadhaya et al., 2013) and the recent IUCN Red List (IUCN, 2016).

Pearson correlations analysis was performed to examine the effect of fragment size and anthropogenic disturbances on species richness, density, basal area and various diversity indices. The software SPSS statistical package (Version 20) was used for statistical analysis.

Results

Site Characteristics and Disturbances

The fragmented patches under study were scattered amidst degraded grassland and human habitation. The major threats to the existing remnant patches are anthropogenic disturbances. Encroachment of forest land for agriculture, settlement, mining of coal, construction of ponds around streams and sand mining are common sight around most of the patches. Timber and non-timber forest product extraction, browsing, rampant fire during winter are also threatening the very existence of these remnant patches (*Table 1*). The number of cut stumps was high in S2 (105 stems) followed by M3 (94) and L1 (90) as shown in *Table 2*. Forest fragments M3, L1, S1 and S3 were highly disturbed whereas, S2, M1, M2, L3 and VL3 were mildly disturbed. The remaining three forest fragments L2, VL1 and VL2 were least disturbed. The patches close to human habitation were more disturbed (*Table 1*).

Floristic Composition and Diversity

A total of 160 woody species (≥5cm dbh) belonging to 105 genera and 54 families were enumerated from all the studied fragments. The average species richness per patch was 75 ±2. The species richness in the fragments ranged from 60 species in S2 to 83 in M1, L2 and VL1 fragments. The mean number of species was 69 species in Small Fragment class(S), 75 in Medium (M), 76 in Very Large (VL) and 77 in Large (L) classes (*Table 2*). The species richness did not increase with the increase in patch area ($r = 0.16$, $p = 0.61$). The studied fragments had a number of rare, endemic and threatened species (*Appendix 1*). Overall, the family Rubiaceae had the highest number of genera (8) followed by Lauraceae (7) and Rutaceae (6). In terms of species richness, Fagaceae was the dominant family with 17 species, followed by Rubiaceae (13) and Lauraceae (11).

Diversity indices varied among different fragment classes. The mean Shannon Wiener's diversity index (H') values ranged from 3.54 in Small to 3.76 in Medium fragment, while Large and Very Large showed a value of 3.56 and 3.64 respectively. Based on the H' values the fragment classes can be arranged in the order of

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M>VL>L>S. The Simpson's dominance index (D) showed a reverse trend to that of H'values. Based on the dominance index the fragments can be arranged in the order of S=L>VL>M. The values ranged from 0.03 to 0.05. Pielou's eveness index (J) ranged from 0.82 to 0.87 in all the fragments (*Table 2*).

Table 2. *Species richness, density, basal area and diversity indices (per 0.4 ha) in different fragments*

Fragment Category	Species Richness	Density	Basal Area (m^2)	H'	D	J	No. of Cut stumps
S1	77	670	17.41	3.74	0.034	0.86	39
S2	60	620	17.47	3.41	0.052	0.83	105
S3	72	651	20.17	3.46	0.056	0.81	53
Mean	69±5	647±15	18.35±0.91	3.54±0.1	0.05±0.01	0.83±0.01	66
M1	83	663	16.84	3.92	0.025	0.88	49
M2	79	612	19.35	3.84	0.028	0.88	55
M3	67	743	16.47	3.53	0.046	0.84	94
Mean	75±5	673±38	17.55±0.9	3.76±0.1	0.03±0.01	0.87±0.02	66
L1	75	713	15.82	3.59	0.052	0.84	90
L2	83	501	21.02	3.74	0.039	0.84	24
L3	72	628	20.7	3.35	0.07	0.78	30
Mean	77±3	614±62	19.18±1.7	3.56±0.1	0.05±0.01	0.82±0.02	48
VL1	83	690	19.95	3.86	0.029	0.87	10
VL2	69	597	20.85	3.41	0.053	0.80	62
VL3	76	626	21.68	3.66	0.041	0.84	22
Mean	76±4	637±27	20.83±0.5	3.64±0.1	0.04±0.01	0.84±0.02	31

Similarity

The species composition of the fragments in the study sites showed high similarity index ranging from 52-77% (*Table 3*). The similarity percentage between L1 and VL3 was highest (77%) and the least between VL2, M2 and M3 (mean 52.5%). The mean percentage of similarity among the fragment classes are 67% for Small, 63% for Medium, 69% for Large and 65% for Very Large respectively (*Table 3*).

Table 3. *Sorensen's similarity index (%) among the different fragments*

	S1	S2	S3	M1	M2	M3	L1	L2	L3	VL1	VL2	VL3
S1	1											
S2	66	1										
S3	67	68	1									
M1	68	59	66	1								
M2	64	65	68	59	1							
M3	68	60	69	66	63	1						
L1	67	71	68	61	66	68	1					
L2	75	66	63	65	67	69	71	1				
L3	72	61	61	57	56	61	69	68	1			
VL1	68	64	61	60	64	60	65	71	68	1		
VL2	67	57	61	58	52	53	60	58	62	67	1	
VL3	63	66	66	67	67	63	77	68	59	63	66	1

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Density and Basal Area

A total of 7711 individuals were enumerated in 4.8 ha sampled area. The stem density of woody individuals ranged from 501 individuals per 0.4 ha in L2 to 743 in M3 (*Table 2*). There was no significant increase in the stand density (r = -0.06, p= 0.86) with the increase in patch size (*Table 4*). However, the density increased significantly with the increase in disturbance (r = 0. 71, p= 0.01). The dominant species across the fragments in terms of density includes *Castanopsis purpurella, Helicia nilagirica, Persea odorratissima, Quercus semiserrata* and *Syzygium tetragonum* (*Annexure 1*).

The basal area showed a gradual increase with increase in patch size (r = 0.61, *p*= 0.01) but decreased with the increase in disturbance (r = -0.74, p= 0.01) (*Table 4*). The values were low (*Table 2*) in Medium (17.55 ± 0.90m^2/0.4ha) and Small (18.35 ± 0.91) as compared to Large (19.18 ± 1.70) and Very large (20.83 ± 0.46) patches. In terms of basal area, the dominant species in different fragments sizes are *Castanopsis purpurella*, *C. tribuloides*, *Helicia nilagirica*, *Quercus semiserrata* and *Syzygium tetragonum*.

***Table 4.** Pearson's correlation between fragment size, disturbance and different phytosociological attributes*

Parameters	Area	Disturbance index	Species richness	Density	Basal area	H'	D	J	Cut stumps
Area	1								
Disturbance index	-0.51	1							
Species richness	0.16	-0.36	1						
Density	-0.06	0.71**	-0.18	1					
Basal area	0.61*	-0.74**	0.19	-0.65*	1				
H'	0.04	-0.24	0.85**	0.01	-0.12	1			
D	-0.07	0.25	-0.67*	-0.02	0.11	-0.95**	1		
J	-0.04	-0.10	0.62*	0.15	-0.31	0.94**	-0.97**	1	
Cut stumps	-0.43	0.67*	-0.47	0.44	-0.43	-0.40	0.34	-0.25	1

* Significant at $p < 0.05$ and ** $p < 0.01$

Population Structure

The distribution of density in different diameter classes exhibits a reverse J-shaped curve. The number of individuals was concentrated in the lower diameter class (5-15 cm dbh) that gradually decline with the increase in diameter classes (*Figure 4*). The percentage contribution to total density by individuals in 5-15cm diameter-class ranged from 55-74% while the density in upper diameter-classes (>46 cm) contributed to about 1.35-13%. S1 contain the highest percentage (74%) of individuals in the lower diameter-class (5-15cm) followed by M3 (73%), L1 (72), VL1 and VL2 (71 each), M1 (67), S2 and L3 (65 each), S3 (64), VL2 (62), M2 (56) and minimum was observed in L2 (55). VL3 had the highest number of individuals in the higher diameter class (>66cm) with 12 stems per 0.4ha. The stem density in the lowest diameter class (5-

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15cm) was highest in the sites corresponding to fragments with maximum disturbance score viz., M3 (542 individuals), L1 (510) and S1 (496). There was a total absence of individuals in >66cm dbh class in L2 (*Figure 4*).

Basal area distribution exhibited a very irregular trend among the different diameter classes and fragment sizes. The basal area was high in 16-25cm diameter class in all the fragments that gradually decreased till 56-65cm dbh class followed by an increase in > 66cm dbh class in all the stands except in S2, M3 and L1 (*Figure 4*). The percentage contribution to the total basal area by the lower diameter class (5-15cm) was highest in M3 (26%) and lowest in VL1 and VL2 (12% each). Similarly, the contribution to basal area by >66cm dbh class was highest in larger fragment classes i.e., VL3 (39%), VL1 and VL2 (20% each).

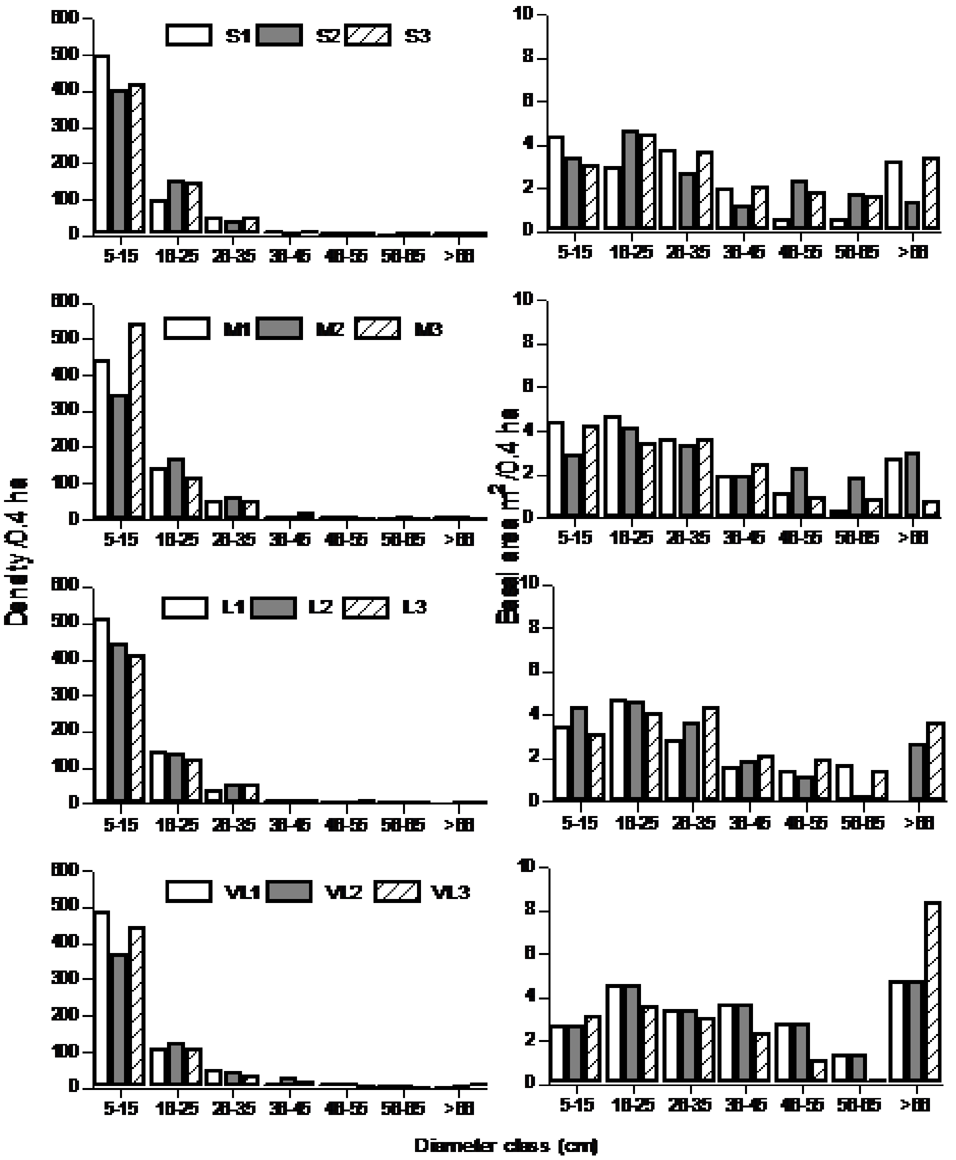

Figure 4. *Distribution of density and basal area in different diameter classes in the studied fragments*

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Discussion

The site characteristics of the studied fragments have shown that they are exposed to one or the other form of human disturbances. Forest fragmentation and anthropogenic disturbance can cause synergistic detrimental effect on the composition and structure of a plant community. The presence of 160 woody species belonging to 105 genera and 54 families indicates a considerable level of plant diversity in the study area. It seems that the variation in species richness among the fragments is due to land use history, geographical proximity to human habitation, and anthropogenic disturbances (Echeverria et al., 2007). The species richness showed no linear relation with the increase in patch size. This finding is similar to that reported from temperate forest of Chile (Echeverria et al., 2007) and tropical highlands of Chiapas, Mexico (Cayuela et al., 2006; Ochoa-Gaona et al., 2004). Such observation could be attributed to spatial shape complexity, dispersal mechanism, establishment and maintenance requirements of the species (Lord and Norton, 1990; Bierregaard et al., 1992). Isolation could be another important determinant of tree diversity in the fragments. The presence of patches close to each other might have favoured the recruitment of species. Protection and management may be one of the reasons for fair presence of species as observed in small fragments (S1 and S2). However, these small fragments are under severe threat due to illegal extraction and other human disturbances. Edge effect as well as human disturbance favoured pioneer species like *Castanopsis purpurella*, *Helicia nilagirica*, *Quercus semiserrata*, etc. Such species constituted 66-89% of the total density and were dominant in all the fragments. Several studies have reported a preponderance of pioneer species after disturbance (Aguilar-Santaleiss and Castillo, 2013). The high similarity in species composition (52-77%) among the studied fragments could be the close proximity of the patches to each other. Trees are long lived species and hence time lag may delay the impact of fragmentation on species composition shift among the fragments (Tabarelli et al., 2004).

The studied forests harbour a number of rare (*Acer laevigatum*, *A. oblongum*, *Cinnamomum pauciflorum*, *Croton oblongus*, *Cyathea gigantea*, *Engelhardtia roxburghii*, *Litsea leata*, *Photonia integrifolia*, *Podocarpus neriifolia*) and endemic species (*Camellia cauduca*, *Citrus latipes*, *Ilex embeloides*, *I. venulosa*, *Schima khasiana*, *Viburnum simonsii*) of the region. These remnants patches are also the natural habitat to many of the species threatened at global level (*Eleocarpus prunifolius*, *Engelhardtia spicata*, *Ilex venulosa*, *Magnolia insignis*, *M. punduana*). Therefore, these patches are important from conservation point of view (*Appendix 1*).

Shannon Wiener's diversity index showed no significant increase with area, but was negatively related to dominance index. High dominance of certain species in response to disturbances may be responsible for this behavioural pattern of the community. The negative relation of evenness and dominance index with area indicate a more diverse plant community especially in the smaller fragments. Such findings reveals the potential role of small fragments in conserving plant diversity in fragmented landscape (Toeldo-Aceves et al., 2014; Hernandez-Ruedas et al., 2014).

Though stem density showed no linear relationship with the fragment size, it increased significantly with disturbance. Increase in density with disturbance, reflects the resilience of the fragments following past disturbance. Recruitment of individuals in gaps created due to selective extraction may lead to an increase in stem density as observed in tropical forests of Kenya (Fashing et al., 2004) and India (Bhuyan et al., 2003). According to Atkinson and Marin-Spotta (2015), disturbed forests have high

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density and low basal area and are a characteristic feature of early successsional forest. Further, due to edge effect, there is a preponderance of fast growing pioneer species that often have high ecological amplitude to survive and compete for resources in fragmented patches (Laurance et al., 2004). The physical factors at the edge are known to restructure the plant community (Tomimatsu et al., 2015). An increase in the number of cut stumps and stem density and a decrease of basal area in the smaller fragment categories indicates that such patches are not only vulnerable to edge effect alone but also to anthropogenic encroachment.

The density- diameter distribution showed reverse J-shaped curve and indicates that the studied stands are regenerating. Majority of the individuals (60-70%) were present in the lower diameter class (5-15cm dbh). The proportion of individuals in the lower diameter class (5-15cm dbh) was high in the disturbed sites. Recruitment of light tolerant species might have increased the stem density of the stand following disturbance (Majumdar et al., 2014). The low density in Very Large category may be due to thinning effect caused by larger trees as reported from the sholas of Western Ghats (Mohandass and Davidar, 2010). Extraction of trees for small timbers and poles were responsible for lower density in the middle diameter classes. Other causes could be the cultural disturbances in the form of selective felling during religious festivals and cremations (Jamir et al., 2006; Upadhaya et al., 2008).

There was a positive relation of basal area with patch size due to retention of some old growth large trees. Similar results have been reported from other subtropical forest of the region (Tripathi et al., 2010), tropical rainforest of Western Ghats (Bhat et al., 2000) and south eastern Madagascar (Ingram et al., 2005) and temperate forest of Southern Chile (Echeverria et al., 2007). Reduction of basal area in the smaller fragments represents a modification in the stand structure as the forest had returned to an earlier successional stage (Echeverria et al., 2007). Though the number of individuals was high in the lower diameter class (5-15cm) but their contribution to basal area was low. The irregular distribution of basal area in different diameter classes could be attributed to selective extraction by the villagers.

Conclusion

The forest patches under study are of high conservation value due to its geographical location being in the Indo-Burma biodiversity hotspot (Mittermeier et al., 2004). The area houses a rich plant wealth and has been identified as a high priority area for conservation that lies outside protected area network in Meghalaya (Upadhaya et al., 2013). The study also highlights the potential role of small patches in conserving plant diversity of the region. Thus a network of patches could serve as habitat for subset populations of the original habitat that can interact and exchange resources. They may also act as corridor for various ecological interaction, serves as source of propagules for regeneration and aid in the successional process of the surrounding non forested matrix. Therefore, it may be suggested that human disturbances operating in the area needs to be reduced and the entire landscape be covered under the protected area network. We also suggest that other life forms such as shrubs, herbs, epiphytes should also be considered to understand the overall effect of fragmentation on plant diversity at landscape level.

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APPENDIX

Appendix 1. *List of woody species and their density (per 0.4ha) in the studied fragments*

Species	**Family**	**Fragment classes**											
		Small			**Medium**			**Large**			**Very Large**		
		S1	**S2**	**S3**	**M1**	**M2**	**M3**	**L1**	**L2**	**L3**	**VL1**	**VL2**	**VL3**
**Acer laevigatum* Wall.	Aceraceae	9	4	1	2	3		3	1	1	3	1	5
**Acer oblongum* Wall.	Aceraceae	-	-	-	-	-	-	-	2	3	-	-	-
Acronychia pedunculata (L.) Miq.	Rutaceae	-	1	-	9	8	2	14	11	7	1	2	9
Adinandra sp.	Theaceae	-	-	-	1	-	-	-	-	2	2	1	1
*^*Agapetes variegata* (Roxb.) D. Don.	Myrsinaceae	-	-	-	3	-	-	1	-	-	-	-	-
Albizia chinensis (Osb.) Merr.	Euphorbiaceae	1	-	-	-	-	-	-	-	-	-	-	-
Alyxia fascicularis Benth.	Apocyanaceae	-	-	-	1	-	-	-	-	-	-	-	-
Antidesma khasianum Hk.f.	Euphorbiaceae	-	-	-	-	-	-	-	-	1	1	-	1
^*Ardisia griffithii* Cl.	Myrsinaceae	-	2	1	-	3	-	1	1	-	-	-	1
Ardisia virens Kurz	Myrsinaceae	3	-	-	-	-	-	-	-	-	-	-	-
*^*Beilshmiedia assamica* Meissn.	Lauraceae	3	1	4		3	6	3	1	1	3	-	1
Betula alnoides Buch.-Ham. ex D.Don	Betulaceae	-	-	-	4	-	-	-	-	-	1	-	-
Casearia glomerata Roxb.	Flacoutiaceae	2		4	1	1	-	-	6	4	2	1	
Callophyllum polyanthum Choisy	Clusiaceae	10	-	-	1		6	2	2		23		2
^*Camellia cauduca* Cl.ex Brandis	Theaceae	4	9	4	16	39	14	11	18	21	5	12	22
Camellia caudata Wall.	Theaceae	5	8	1	2	15	-	7	-	5	1	2	12
Capparis assamica Hk.f. & Th.	Capparaceae	-	9	2		-	-	1	-	1	-	-	-
Castanopsis armata Spach.	Fagaceae	13	16	3	21		8	11	7	8	30	29	13
Castanopsis kurzii (Hance) Biswas	Fagaceae	17	20	7	25	11	2	11	3	9	19	22	27

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Castanopsis purpurella (Miq.) Balak.	Fagaceae	49	42	12	25	25	20	5	19	52	42	33	37
Castanopsis tribuloides (Sm.) DC	Fagaceae	30	29	30	21	51	16	20	9	7	28	52	37
Cinnamomum bejolghota (Buch.-Ham.) Sweet.	Lauraceae	-	-	-	-	-	1	1	-	1	1	-	-
**Cinnamomum pauciflorum* Nees	Lauraceae	-	-	-	-	-	-	-	-	-	1	2	-
Cinnamomum tamala Fr. Nees	Lauraceae	20	5	2	17	3	17	14	10	5	14	4	25
Cinnamomum zeylanicum Blume	Lauraceae	10	1	1	-	1	2	1	6	2	1	1	9
^*Citrus latipes* (Swingle) Tanaka	Rutaceae	-	-	-	2	-	-	-	-	-	-	-	-
Clerodendrum bracteatum Wall.ex Walp.	Verbenaceae	-	-	-	-	-	2	-	-	-	-	-	-
Clerodendrum colebrookianum Walp.	Verbenaceae	1	-	-	-	-	-	-	-	1	1	1	-
Clerodendrum wallichii Merr.	Verbenaceae		-	-	-	-	-	-	1	-	-	-	-
^*Coffea khasiana* Hk.f.	Rubiaceae	-	-	-	-	-	-	-	-	-	1		1
**Croton oblongus* Burm.f.	Euphorbiaceae	3	-	-	3	5	-	-	1	1	4		1
**Cyathea gigantea* (Wall.ex Hook.) Holtt.	Cyatheaceae	1	-	1	4	-	5	-	-	-	-	-	-
Daphne involucrate Wall.	Thymelaeaceae	-	3	-	1	-	-	-	2	-	-	1	4
**Daphniphyllum himalayense* (Benth.) Mull. Arg.	Daphniphyllaceae	1	-	-	1	-	-	-	2	3	3	1	2
Decaspermum paniculatum (L.)Kurz.	Myrtaceae	-	-	3	3	1	5	-	-	2	-	-	-
Diospyros kaki Thunb.	Ebenaceae	1	-	1	2	-	3	5	1	3	-	-	-
Dysoxylon gobara (Buch.-Ham.) Merr.	Meliaceae	-	-	-	-	1	-	-	-	-	-	3	-
Elaeagnus latifolia Hk. f.	Elaeagnaceae	-	-	2	-	-	-	-	-	-	-	1	-
Elaeocarpus lanceifolius Roxb.	Elaeocarpaceae	2	1	2		7	3		3	-	-	-	5
#*Elaeocarpus prunifolius* Wall.ex Mull.Berol.	Elaeocarpaceae	29	27	3	15	5	13	4	13	4	10	6	2
Embelia ribes Burm.f.	Myrsinaceae	-	-	-	-	1	-	1	1	-	-	-	-
**Engelhardtia roxburghiana* Wall.	Juglandaceae	-	-	-	1	8	-	3		3	-	-	1
#*Engelhardtia spicata* Lechen ex Bl.	Juglandaceae	1	4	10	-	3	-	7	2		12	1	3
Eriobotryo bengalensis Hk.f.	Rosaceae	6	-	-	2	-	-	-	-	-	-	-	-

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*^*Erythroxylon kunthianum* Wall. ex Kurz	Erythroxylaceae	4	7	8	5	1	2	16	4	10	13		2
Euonymus attenuatus Laws.	Celastraceae	-	-	1	-	-	-	2	2	-	1	-	-
**Euonymus bullatus* Wall. ex Laws	Celastraceae	-	-	-	1	1	1	-	-	-	-	-	-
Euonymus theifolius Wall. ex Laws	Celastraceae	1	-	-	-	-	1	1	-	1	-	-	-
Eurya accuminata DC.	Theaceae	-	-	-	-	-	-	-	1	2	-	3	3
Eurya japonica Thunb.	Theaceae	5	13	53	25	15	15	22	1	9	11	7	26
Exbucklandia populnea (R.Br. ex Griff.)	Hamamelidaceae	1	-	-	5	1	-	-	3	-	6	8	11
Ficus hirta Vahl	Moraceae	-	1	-	-	-	-	-	1	-	1	-	-
Ficus nerifolia Sm.	Moraceae	-	7	-	-	2	-	1	1	-	-	-	1
*^*Fraxinus floribunda* Wall.	Oleaceae	1	1	1	-	-	1	5	8	1	2	1	-
Garcinia anomala Planch. & Triana	Clusiaceae	2	-	-	-	-	1		1	-	9	-	13
Garcinia cowa Roxb. ex DC.	Clusiaceae	30	10	1	2	-	-	3	2	4	7	-	3
Garcinia morella Desr.	Clusiaceae	-	-	1	-	5	-	2	-	-	-	1	-
^*Glochidion thomsonii* Hk.f.	Euphorbiaceae	12	7	1	1	4	7	-	7	6	14	2	8
Glycosmis cymosa (Kurz) Narayan	Rutaceae	1	13	2	31	51	7	11	29	19	-	1	-
Helicia nilagirica Bedd.	Proteaceae	43	36	44	23	29	82	66	13	70	29	99	36
Heteropanax fragrans (D.Don.) Seem.	Araliaceae	-	-	-	-	-	-	-	-	2	3	-	-
Hiptage bengalensis (L.) Kurz	Malphigiaceae	3	-	-	-	-	-	-	-	1	-	-	-
Hyptianthera stricta (Wild.) Wt. & Arn.	Rubiaceae	-	1	-	1	-	-	-	-	-	-	-	-
*^*Ilex embeloides* Hk.f.	Aquifoliaceae	10	7	3	3	4	1	1	1	1	6	2	2
^*Ilex excelsa* (Wall.) Hk.f.	Aquifoliaceae	1		3	5	7	-	6	-	-	2	2	2
Ilex odorata Buch.-Ham ex D.Don	Aquifoliaceae	25	19	5	3	5	1	2	1	3	2	2	3
Ilex sp.	Aquifoliaceae	-	-	1	-	-	-	-	-	-	-	2	-
*^#*Ilex venulosa* Hk.f.	Aquifoliaceae	11	-	3	26	4	7	16	5	-	4	1	2
Itea macrophylla Wall.	Iteaceae	-	1	-	1	1	-	1	-	-	-	-	-

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Itea chinensis Hk. f.	Iteaceae	3	-	-	-	-	-	-	-	-	-	2	-
Ixonanthes sp.	Ixonanthaceae	-	2	-	-	-	-	-	-	-	1	-	-
Ixora nigricans Wt. & Arn. Pradr.	Rubiaceae	-	-	-	-	-	-	-	-	-	-	-	4
^*Ixora subsessilis* G.Don	Rubiaceae	-	-	-	-	-	-	1	-	-	-	-	1
Lasianthus lucidus Blume	Rubiaceae	-	-	-	-	1	-	-	-	-	-	-	-
Lasianthus sp.	Rubiaceae	-	-	-	-	-	-	-	2	-	-	-	-
**Lasianthus tubiferus* Hk.f.	Rubiaceae	3	-	-	-	2	1	-	-	-	-	-	-
Leea indica (Burm.) Merr.	Leeaceae	2	-	-	3	3	-	-	-	-	3	-	-
Ligustrum compactum Hk.f & Th. ex Brandis	Oleaceae	-	-	1	2	1	-	-	-	-	-	-	-
Ligustrum robustum (Roxb.) Blume	Oleaceae	-	2	17	4	13	-	7	4	2	3		2
*^*Lindera latifolia* Hk.f.	Lauraceae	1	-	-	-	-	-	-	-	-	-	2	-
Lithocarpus dealbatus (Hk.f. & Th. ex Miq.) Rehder.	Fagaceae	-	-	5	12	6	-	1	4	-	-	-	-
Lithocarpus elegans (Bl.) Hatus. ex Soep.	Fagaceae	15	4	7	10	19	12	20	3	3	-	20	4
Lithocarpus fenestratus (Roxb.) Rehder.	Fagaceae	4	-	-	-	-	53	-	33	15	12	1	-
*^*Litsea elongata* (Nees) Hk.f.	Fagaceae	26	1	-	-	-	-	2	1	1	4	3	2
*^*Litsea laeta* Wall.ex Nees	Fagaceae	-	-	11	2	-	12	-	4	3	16	-	1
Litsea salicifolia (Roxb.ex.Nees) Hk.f.	Fagaceae	-	6	3	5	-	5	6	-	5	-	9	21
Macaranga denticulata Muell.- Arg.	Euphorbiaceae	-	-	-	-	-	-	-	-	2	-	-	-
Maesa indica (Roxb.) Wall.	Myrsinaceae	-	-	1	-	1	-	-	-	-	-	-	-
Mahonia pycnophylla (Fedde) Takeda	Berberidaceae	1	5		1		1	3	1	-	-	-	-
#**Magnolia insignis* (Wall.) Bl.	Magnoliaceae	-	3	4	10	2	4	1	3	-	6		3
Magnolia sp.	Magnoliaceae	-	-	-	2	-	-	-	-	-	-	-	-
#**Magnolia punduana* Hk.f. & Th.	Magnoliaceae	-	-	1	18	4	5	3	5	-	4	-	11
Microtropis discolor (Wall.) Arn.	Celastraceae	1	-	-	1	-	-	-	2	-	-	-	-
Miliusa roxburghiana (Wall.) Hk.f. & Th. Th.	Annonaceae	-	-	-	-	-	-	-	-	-	4	-	-

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Millettia pulchra (Benth.) Kurz.	Fabaceae	-	-	1	1	1	-	-	-	-	-	-	-
Morinda angustifolia Roxb.	Rubiaceae	-	1	1	-	-	-	2	-	-	-	-	2
Mucuna sp.	Fabaceae	-	-	-	3	-	-	-	-	-	-	-	-
Mussaenda roxburghii Hk.f.	Rubiaceae	-	-	-	-	1	-	-	-	-	-	-	-
Meyna laxiflora Robyns.	Rubiaceae	-	-	2	-	-	2	2	-	1	-	-	-
Myrica esculenta Buch.-Ham. ex D.Don	Myricaceae	10	10	13	24	20	18	4	12	13	31	49	14
Myrsine capitellata (Wall.) Mez.	Myrsinaceae	-	-	-	-	-	3	2	-	-	-	-	-
Myrsine semiserrata Wall.	Myrsinaceae	4	3	1	8	2	1	3	1	-	3	6	1
Neolitsea umbrosa (Nees.) Gamble	Lauraceae	8	4	2	1	4	-	1	-	-	-	10	4
Neolitsia cassia (L.) Koster.	Lauraceae	-	3	-	-	-	4	14	2	2	17	-	1
Olea dentata Wall.ex DC.	Oleaceae	-	-	-	-	2	-	-	-	-	2	-	-
Persea duthiei (King ex Hk.f.) Koster.	Lauraceae	-	-	-	-	-	-	-	-	3	2	57	2
Persea odorratissima (Nees) Koster.	Lauraceae	2	3	18	31	23	30	20	5	16	11	8	47
Phoebe lanceolata (Nees) Nees	Lauraceae	-	-	3	3	3	2	2	-	-	9	6	7
*^*Photinia arguta* Lindl.	Rosaceae	-	-	1	-	-	-	-	-	-	-	-	-
*^*Photinia cuspidata* (Bertol.) Balak.	Rosaceae	-	-	-	-	-	-	1	-	-	-	3	-
*^*Photinia integrifolia* Lindl.	Rosaceae	-	-	-	6	2	-	-	1	-	1	1	1
Photinia notoniana Wt. & Arn.	Rosaceae	-	-	-	5	3	-	2	1	2	4	1	-
Pinus kesiya Royle ex. Gordon	Pinaceae	-	-	-	-	-	3	-	-	-	-	7	3
Pithocellobium monodelphum (Roxb.) Koster.	Fabaceae	1	1	-	-	1	2	1	1	1	2	1	3
Pittosporum humile Hk.f. & Th.	Pittosporaceae	2	13	20	12	28	49	21	27	25	20	10	14
^*Podocarpus neriifolia* D.Don	Podocarpaceae	-	-	-	1	-	-	-	-	-	-	-	-
Prunus punctata Hk. f. & Th.	Rosaceae	2	1	3	3	2	10	2	1	2	3	4	-
Psychotria adenophylla Wall.	Rubiaceae	-	3		1	4	-	2	4	-	1	-	-
*^*Psychotria simplocifolia* Kurz.	Rubiaceae	3	-	9	3	2	-	-	-	-	-	2	1

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Pyrularia edulis DC.	Santalaceae	1	-	-	-	-	-	-	-	-	1	-	-
Pyrus pashia D.Don	Rosaceae	1	-	-	-	-	-	-	-	-	-	-	-
Quercus sp.	Fagaceae	30	-	-	-	1	36	-	10	20	-	1	3
**Quercus glauca* Thunb.	Fagaceae	13	-	3	-	-	-	1	-	2	7	1	-
Quercus kamroopii D.Don	Fagaceae	9	-	-	-	1	8	-	3	2	3	4	-
Quercus lancifolius Roxb.	Fagaceae	13	-	-	-	1	-	-	1	-	-	1	-
Quercus semiserrata Roxb.	Fagaceae	23	74	89	7	21	71	139	7	109	16	5	13
Randia spinosa (Thunb.) Poir.	Rutaceae		-	-	-	-	-	-	-	-	2	-	-
Randia wallichii Hk.f.	Rutaceae	3	-	-	-	-	-	1	1	1	-	-	-
Rauvolfia densiflora (Wall.) Benth. ex Hk.f.	Apocyanaceae		-	-	-	-	-	-	-	-	-	-	1
Rhus accuminata DC.	Anacardaceae	17	7	15	10	10	26	32	22	8	23	2	2
Sarcococca saligna (D.Don.) Muell.-Arg.	Anacardaceae	-	-	-	-	-	-	-	-	-	2	-	2
*^*Sarcosperma griffithii* Cl.	Sapotaceae	23	3	12	46	14	7	22	14	8	11	3	11
Schefflera glomerulata Li.	Araliaceae	4	-	-	-	-	-	-	1	-	-	-	-
Schefflera hypoleuca (Kurz) Harms	Araliaceae	1	6	5	15	7	3	-	15	1	12	-	3
Schefflera venulosa (Wt. &Arn.) Harms	Araliaceae	6	2	-	-	1	-	12	3	-	7	-	-
*^*Schima khasiana* Dyer	Theaceae	-	2	1	1	2	-	-	-	-	6	2	6
Schima wallichii (DC.) Korth.	Theaceae	5	48	3	11	6	13	22	54	8	36	15	21
Skimmia laureola (DC.) Sieb & Zucc.ex Walp.	Rutaceae	-	1	-	-	-	-	2	-	-	-	-	-
Sterculia roxburghii Wall.	Sterculiaceae	-	5	9	-	4	2	14	4	2	1	-	-
Styrax serrulatum Roxb.	Styracaceae	3	14	12	9	-	9	11	4	16	1	-	-
Symplocos glomerulata King ex Cl.	Symplocaceae	3	-	7	-	-	-	-	1	-	-	-	1
Symplocos javanica (Bl.) Kurz.	Symplocaceae	4	-	1	1	2	28	2	3	3	3	2	10
Symplocos paniculata (Thunb.)Miq.	Symplocaceae	-	-	-	1	-	1	-	-	-	-	-	-
Symplocos spicata Roxb.	Symplocaceae	16	8	22	10	28	4	23	7	7	13	25	18

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Syzygium cumini (L.) Skeels	Myrtaceae	-	29	34		11	-	-	-	-	1	4	10
Syzygium macrocarpum (Roxb.) Balak.	Myrtaceae	25	-	-	2	1	32	-	5	35	16	-	-
Syzygium tetragonum Kurz.	Myrtaceae	-	48	68	49	18	16	15	6	26	41	20	27
**Tupidanthus calyptratus* Hk. f. & Th.	Araliaceae	-	-	-	-	-	-	-	-	1	-	-	-
^*Turpinia nepalensis* Wall. ex Wt.&Arn.	Styphyleaceae	-	-	-	1	-	-	-	-	-	-	-	-
Uncaria sp.		-	-	-	-	-	-	-	-	-	-	1	-
Vaccinium sprengelii (G.Don) Rehd.	Ericaceae	-	-	1	3	5	1		1		2		4
Vernonia volkameriifolia DC.	Asteraceae	2	3		3	3	1	1		1	-	-	4
Viburnum coriaceum Bl.	Caprifoliaceae	6	-	-	-	-	-	-	1	3	-	1	-
Viburnum odorratissamum Ker.	Caprifoliaceae	-	-	-	-	-	-	-	2	-	3	-	-
^*Viburnum simonsii* Hk.f. & Th.	Caprifoliaceae	-	-	19	2		12	-	1	-	-	-	-
Wendlandia wallichii Wt. & Arn.	Rubiaceae	3	-	2	3	1	4	12	16	3	5	1	7
Zanthoxylum armatum DC.	Rutaceae	-	-	-	2	1	-	-	-	-	-	-	-
Unidentified sp 1	Apocyanaceae	-	-	-	-	-	-	-	-	-	-	-	1
Unidentified *sp 2*	Anacardaceae	-	-	-	-	-	*1*	-	-	-	-	-	-

'-' = Indicates absences; '*' = Rare; '^' = Endemic; '#' = Threatened

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SELF-SUSTAINING FOREST

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Abstract. It has been recognized and discussed in the literature that values associated with forest integrity, i.e., sustainability and diversity, are relevant to the three aspects of self-organisation: resilience, health, and evolvability. However, evolvability has not yet been studied and also there is still no relevant notion of forest health. This calls for a biodynamic approach to forests and clearly addresses questions relating to scaling and self-organisation since the different levels of detail must be compatible to ensure the consistency of sustainability and diversity assessments. To support the development of a biodynamic approach to forests we review the concepts of self-organisation, criticality and resilience, and their relationship to forest integrity in the light of fractal organisation theory. An exploration of fractal connectivity behind key bioindicators and biomonitors in the avoidance of a biodiversity threshold can be suggested thus far. Forest integrity refers to empirical scaling relationships that are emergent features of biodiversity. In a forest, species as different as fungi, plants, animals and insects, and processes as disparate as disturbance, dispersal, facilitation/competition and nutrient cycling, are related through ecological interaction networks; a 'fractal' is the 'collective phenomenon' of these networks.
Keywords: *biodynamic approach, forest integrity, fractal connectivity*

Introduction

Cyclic processes in the biosphere are self-organised and demonstrate sustainable development that inevitably involves change (Kazansky, 2010; Kimmins et al., 2007). The most important direct driver of change in forest biodiversity and ecosystems is habitat shifts and forest loss. Nearly half of the world's original forest cover has been lost, yet the economic potential of a large part of the global forest area is under-realized because of a lack of sustainable forest management (FAO, 2014). Even if forest loss were to end today, it would take hundreds of years for species numbers to reach a new, lower, equilibrium in response to the habitat shifts that took place in recent centuries (Millennium Ecosystem Assessment, 2005). This means that since large areas are required to conserve viable populations and that since nature reserves eventually become isolated islands in the landscape, the long-term fate of many forest-dependent organisms will depend on activities and conditions in the unreserved portions of forests (Lindenmayer and Franklin, 2002; Whittaker et al., 2001; Kemp, 1992). For this reason, the present understanding of sustainable forest management must include values associated with integrity. A significant change, compatible with economic realities, requires the intrusion of biological considerations and conservationist philosophy into modern methods of exploitation (Stern and Roche, 1974). This calls for a biodynamic approach to forests and clearly addresses questions relating to scaling and self-organisation since the different levels of detail must be compatible to ensure the consistency of sustainability and diversity assessments.

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Values associated with integrity, i.e., sustainability and diversity, are in essence about maintaining the combined biospherical-societal system: humans, with their social, cultural, economic, and environmental needs, are an integral part of ecosystems. It means that the capacity to sustainably use forests rests on our understanding and interpretation of pattern and process at several scales, the recognition of thresholds, and the ability to translate knowledge into appropriate management actions in a reflexive manner (Garmestani and Benson, 2013; Thompson et al., 2009). However, there is no single underpinning model that fully meets all the requirements for evaluating the sustainability of multi-functional forest management (Rennolls et al., 2007). So, the focus of attention shifts from the value components of sustainable development projects, to the domain of existence, in that we can map and model single, or even multiple relationships, but not a total set of evolving interactions and feedbacks (Fernandez et al., 2014; Jiggins and Röling, 2002; Waldrop, 1992). Here the reflexivity makes possible to connect any aspects of reality, setting up feedback loops between them (Soros, 2010). This, in turn, makes fractal organisation theory, inspired by systems theory, fractal geometry, quantum mechanics, information dynamics, sociobiology, epigenetics, evolutionary biology and game theory, the central subject herein (Raye, 2012).

Fractal organisation theory could illuminate evolving interactions and feedbacks from the multiple perspectives of different types, so it is a means to support the development of underpinning models which are consistent, and scale appropriately across the levels of self-organisation and conceptual frameworks. Forest integrity is about three aspects of self-organisation: resilience, health, and evolvability (Freedman, 2012; Kay and Regier, 2000). Evolvability, however, has not yet been studied because it was presumed at the outset: empirical experiences with quantitative genetics and selective breeding produced a consensus that ‘phenotypic variation was effectively like a gas which could flow into any selective bottle’ (Luo, 2014; Altenberg, 2014). Also, there is still no relevant notion of forest health, as there is for humans: a set of properties that have been selected through evolution because they maximize fitness (De Leo and Levin, 1997). Resilience is ‘the capacity to change in order to maintain the same identity’ (Folke et al., 2010). Accordingly, to support the development of a biodynamic approach to forests we review the concepts of self-organisation, criticality and resilience, and their relationship to forest integrity in the light of fractal organisation theory.

Scaling laws

Pattern integrity or self-similarity – the retention of distorted copies of itself across scales – is a typical property of fractals, a concept introduced by Benoît Mandelbrot (1924-2010) and one of the fundamental mathematical results of the 20th century (Satija, 2016; Raye, 2012; Rozenfeld et al., 2009). Fractals are often considered the ‘fingerprints of chaos’: the term ‘fractal’ is based on the Latin *frāctus*, derived from *frangere* which signifies to break, to create irregular fragments (Mandelbrot, 1983). Also, a fractal is known as expanding symmetry or evolving symmetry (Kumar et al., 2017). Self-similarity is symmetry across scale. The manner, in which a fern leaf’s overall shape is replicated in each of its leaflets, and again in the subleaflets of each leaflet, is a familiar illustration of fractal relationship (Mosko, 2010). However, fractals are not extrapolated from a geometric logic based on units. Rather, we can think of

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fractals as processes, possessing a self-replicating basis, which lead to a non-integer dimensionality (Fielder and King, 2014). Let's take, for example, the branching structures of resource distribution networks, such as the xylem that transport water through plants. According to West et al. (2009), 'the entire forest is, in a very real sense, a hierarchically branching resource supply network that can be described mathematically and behaves structurally and functionally like a scaled version of the branching network of the trees it contains': the analysis of the branching size distribution reveals an exponent which is essentially identical to the tree size distribution within a forest. So, a 'fractal' is what 'emerges' from these networks; it's the 'emergent property' of the network's memory. The analysis of fractality can provide a new strategy for studying cellular, organismal and community differentiation since fractal dimension is a good quantitative measure of the degree of morphological differentiation; it is also a useful measure for comparative studies across and among species, as they relate to cellular evolution (Smith and Behar, 1994). It should be noted, however, that the existence of changes in fractal dimension when shifting between scales implies that in place of true pattern integrity, we observe only partial self-similarity over a limited range of scales separated by transition zones. Fortunately, tests carried out using multifractals, which are objects that need a continuous spectrum of exponents to be described, may disclose the properties encoded in the data relating to the relationships on different scales; the basic graphic tools here are known as multifractal spectra or spectra of singularity (Drożdż and Oświęcimka, 2015). On the other hand, real fractals are, in fact, multifractals: the measure is not the same in every subset, and each of them has a different fractal dimension and a different associated diverging exponent (Solé and Manrubia, 1995). Several good introductions to multifractal methods applied to ecology can be found in Scheuring and Riedi (1994) and Borda-de-Água et al. (2007) (Saravia, 2014). Multifractals have been applied to vegetal communities (Scheuring and Riedi, 1994), tropical rainforest (Manrubia and Solé, 1996), and to the characterisation of species-area relationships (Laurie and Perrier, 2011; Yakimov et al., 2008; Borda-de-Água et al., 2002). Borda-de-Água et al. (2007) used the so-called multifractal approach to show that models which assume symmetric neutral dynamics and use realistic dispersal kernels predict scaling patterns of diversity and distribution very similar to those observed by Hubbell and Foster (1983) in the Barro Colorado Island rainforest, in Panama. Solé and Manrubia (1995) constructed a simple cellular automata model in order to simulate the gap dynamics of the Barro Colorado Island rainforest as well as the observed macroscopic spatial regularities. The observed and simulated fractal behaviour was shown to be related to self-similar dynamics of biomass.

'From a wildlife perspective, each organism scales the environment differently, and thus there is no absolute size for a landscape' (Sun and Southworth, 2013). As scale changes, new patterns and processes may emerge and controlling factors may shift even for the same phenomena (Wu and Li, 2006). Fortunately, the fractal hierarchy is a method which can be used to unify different scaling phenomena and rules in complex systems (Seuront, 2009). 'The hierarchy always follows a pair of exponential laws and a power law' (Chen, 2012). Power laws describe empirical scaling relationships that are emergent quantitative features of biodiversity (Brown et al., 2002). A power law is obtained when one observes a straight line in a plot of 'the number of events' versus 'how often they occur'; in other words, the probability $f(x)$ of an event of magnitude x occurring is inversely proportional to x: $f(x) \sim x^{-\alpha}$ (Rhodes and Anderson, 1996). $f(x) \sim$

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x^{-1} – a critical dependence – is often associated with ‘self-organised criticality’ which provides a general mechanism for the emergence of scale-free networks with the characteristic power-law distribution of links (Graham, 2014; Nottale, 2013; Laurienti et al., 2011; Messier and Puettmann, 2011; Turcotte, 1999; Bak et al., 1989). ‘Scale-free model’ incorporates two generic mechanisms thought to be common to many real-world networks: growth of the network by addition of nodes and links at each time step and preferential attachment of new nodes to certain highly connected hubs – the existing nodes with a high number of links. In other words, ‘A scale-free topology automatically emerges whenever new species (nodes) add preferentially to pre-existing ones with a probability proportional to the number of pairwise interactions (links) of the target species’ (Jordano et al., 2003). However, most ecological interaction networks examined so far have cut-off numbers of pairwise interactions per species giving rise to a gradient of variation from scale-free to broad-scale and to single-scale distributions; these distributions depart in most cases from the power-law beyond cut-off values. Constraints in the addition of links such as morphological mismatching or phenological uncoupling between mutualistic partners restrict the number of plant-animal interactions established, causing deviations from scale invariance, which is solely described by a power-law function, $f(x) = kx^{\alpha}$, where the power-law exponent, α, is a measure of scale-invariance, and k is a constant (Katz, 2016). In food webs, the distribution of links changes from (partial) power-law to exponential to uniform as the level of connectance increases (Dunne et al., 2002).

Self-organised phenomena

Nothing happens directly in this indirectly ordered universe (Schauberger, 1936). Unlike the action of seasons and natural disasters, long-term change in the composition of communities is brought about by the activities of living organisms which themselves inhabit the environment. Over a period of time, the environment is modified by these organisms so it becomes suitable for colonization by another species and less suitable for those already there (Rose, 2005). For example, after a stand-replacing disturbance, shade-intolerant species colonize and grow into a dominant canopy, but due to their shade-intolerance they are unable to regenerate under their own canopy, so the understory (composed of shade-tolerant species) gradually replaces the canopy (Kotar, 1997). Thus, all elements of any developing living system co-determine each other, whether it is the coevolution of biological species, a behavioural act or an immune response (Kazansky, 2015). Any living organism has relatively autonomous organisation of metabolic processes and, at the same time, all living creatures are fundamentally dependent on each other via trophic, behavioural or sexual relationships, and also indirectly, via the environment (Levchenko et al., 2012). A forest exists by virtue of all the fungi, trees, other plants, insects, birds and other animals, and they are fully what they are by virtue of dwelling in that forest; neither can ‘exist’, at least not fully, without the other.

‘Self-organisation is basically the spontaneous creation of a globally coherent pattern out of the local interactions between initially independent components’ (Heylighen, 2001). ‘In the optic of biological research, the common meaning of self-organisation is defined by the global emergence of a particular behaviour or feature that cannot be reduced to the properties of individual system’s components such as molecules, agents and cells’ (Camazine et al., 2003 as cited in Di Marzo Serugendo et al., 2011).

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'Physiological interactions among molecules, cells, tissues, organs do not simply sum each other up: they are "entangled", "non-local", "non-separable" . . . they are "superposed"' (Longo and Montévil, 2011). Therefore, despite its intuitive simplicity as a concept, self-organisation has proven notoriously difficult to define and pin down formally or mathematically, and it is entirely possible that any precise definition might not include all the phenomena to which the label has been applied. One of the objectives of the present article was, therefore, to give prominence to the concepts of self-organised criticality and resilience because they are of relevance to forest integrity. As a result, forest integrity could be understood in terms of attractors as defined by conceptual inferences related to self-organised criticality and resilience (*Table 1*). The explanatory power of self-organised criticality stretches so far as to assume that a given scale-free phenomenon is caused by the system which organises its critical state by itself (Pruessner, 2012). This critical state acts as an attractor. The fractal patterns may be a fingerprint of a system close to a critical point (Manrubia and Solé, 1996). To substantiate such a viewpoint, Manrubia and Solé (1996) performed an extensive study of a real rainforest in Barro Colorado Island, Panama. They found the strong evidence of self-organised critical state in the power laws that the magnitudes of the system follow, both in space (fractality, correlation function, clearings and tree sizes distributions) and time (biomass fluctuations). Moreover, self-organised critical models of extinction have been used to explain power-law distributions of species' life span and extinction events in statistical evidence from the fossil record (Solé et al., 1997; Solé and Bascompte, 1996; Sneppen et al., 1995; Bak and Sneppen, 1993).

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Table 1. *System integrity in terms of attractors by conceptual inferences related to self-organised criticality and resilience*

Concept	**Inference**	**Connection**
Self-organisation	'The basic mechanism underlying self-organisation is the variation which explores different regions in the system's state space until it enters an attractor' (Heylighen, 2001). 'Standard examples of attractors are stable equilibrium and stable limit cycle' (Fradkov and Chen, 2009). A limit cycle of infinite period is sometimes referred to as a chaotic state (Li and Yorke, 1975). An attractor – a region in state space that a system can enter but not leave – is a mathematical model of causal closure. 'Closure usually results from the nonlinear, feedback nature of interactions' (Heylighen, 2001). Therefore, it seems that reflexivity can act as an attractor when attempting to predict the outcome of a self-organising system at work (see Schiavello, 2013; Sandywell, 1996).	Reflexivity as an attractor
Criticality	'Dynamical criticality, a central property for the functioning of a living organism, naturally emerges as a consequence of evolution that favours evolvability' (Torres-Sosa et al., 2012). Also, it is a property of (classes of) dynamical systems that have a critical point as an attractor (Aschwanden, 2011; Bak and Creutz, 1994). Actually, many slowly driven open non-equilibrium systems self-organise to a critical point where everything can happen within well-defined statistical laws (Jensen, 1998; Bak, 1996; Bak et al., 1989). Moreover, as a system parameter changes through a critical value, a symmetry-broken attractor can be born (Lai, 1997). Lastly, when the symmetry is lost, it can be said that it is replaced by a collective mode.	Symmetry-broken attractor
Resilience	An ecosystem is resilient if it remains in the same domain of attraction and returns to the same state after a disturbance (Rietkerk and van de Koppel 2008). However, it may exist almost continuously in a transient state if there is frequent disturbance. So, it turns out that the final attractor toward which the system will converge (e.g., successional pattern, community type, etc.) usually depends on the initial conditions involving several attractors, leading to difficult issues related to the ability to predict which attractor a given trajectory will asymptote from (Freire et al., 2008; McDonald et al., 1985). Hypothetically, intransitivity, i.e., coexistence of attractors, is a peculiar characteristic of meta-communities without strict competitive hierarchies (Freire et al., 2008; Kerr et al., 2002).	Intransitivity of attraction

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The basic assumption of resilience thinking is that systems are most resilient in their natural (evolved) states (Hopkins, 2009). Over the long run resilience is needed to maintain organismal fitness, however, the dynamics of organismal fitness remain poorly understood over long time scales (Wiser et al., 2013). Although researchers such as Kauffman (1993) have started exploring the structure of fitness landscapes for various formally defined systems by computer simulation, examples of individual adaptation via plasticity by temporal variation of fitness-related traits observed during the lifetime of forest organisms are very seldom documented at this time (see Lindner et al., 2008; Heylighen, 2001; Durzan, 1993). This leads to a reconsideration of the traditional approach to forests focused on long-term dynamics in favour of biodynamic approach. 'The commonly accepted fact is that the cell/organism (any living organisation, in fact) is an open nonequilibrium system, which exists and functions only because of the incessant flow of energy/matter passing through it' (Kurakin, 2011). For that reason, one of the most fascinating and mould-breaking findings has been the discovery of self-organised spiral/loop patterns, occurring commonly in nonliving and living nature (Luo and Zhan, 2008; Hill, 2006; Heylighen, 2001; Bascompte and Solé, 1998; Jean, 1994). Spirals exist in formations such as weather patterns because the interplay between physical forces and matter tend towards that shape, while they also exist in formations such as forests. Fractal hierarchy underlies these formations in all growth processes (Hill, 2006; Selvam, 1998). For example, a positive feedback loop that results in periodic organ formation has been recently uncovered behind the spiral patterns of leaves on a stem by Bhatia et al. (2016). According to Selvam (1998), such patterns are the clearest examples of self-organised criticality in the plant kingdom.

Resilience, or the stabilizing effect of feedback loops, is defined as the ability of an adaptive system to absorb impacts before a threshold between attractors is reached where the system changes into a different state altogether (Messier and Puettmann, 2011; Thompson et al., 2009; Heylighen, 2001). Since the reaching of an attractor is an automatic process it can be viewed as a general model of self-organisation. Most modelled systems with just one stable attractor (e.g., successional pattern, community type, etc.) tend to return to this attractor when perturbed in their dynamics. When the dynamical system has more than one coexisting attractor, it often turns out that the fractal boundaries of the basins of attraction are leading to difficult issues related to the ability to predict which attractor will a given trajectory asymptote to (McDonald et al., 1985). 'An observer might see one kind of behaviour over a very long time, yet a completely different kind of behaviour could be just as natural for the system' (Gleick, 2008). This implies intransitivity – a major factor stimulating emergence of chaotic dynamics (Klimenko, 2015; Lorenz, 1990; Crutchfield et al., 1986). Stone and Ezrati (1996) argued that chaos theory may be particularly useful in modelling vegetation change, where nonequilibrium dynamics (e.g., disturbance, natural mosaic cycling, and habitat fragmentation) often prevail (Kenkel and Walker, 1996). Nevertheless, emphasis on a broader understanding of possible system behaviours and the effects of human intervention has contributed to a significant shift toward resilience thinking, away from the mathematics of chaos.

Self-organisation of a self-sustainable ecological community is a highly-ordered non-random process based on information written in the genomes of participating species. 'The genetic program of species constitutes the informational basis for the compensatory environmental processes initiated by the biota when challenged by an environmental change' (Gorshkov et al., 2004). In a forest, species as different as fungi,

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plants, animals and insects, and processes as disparate as disturbance, dispersal, facilitation/competition and nutrient cycling, are related through ecological interaction networks; a 'fractal' is the 'collective phenomenon' of these networks. Not coincidentally, loss of fractal dimension by a system implies loss of collectivity, i.e., capability of the interconnected components to interact in a common mode (Waliszewski et al., 1999). Moreover, if fractal space, in which a dynamic process takes place, becomes a classic, i.e., Euclidean, space with integer dimension, this means that the process has left its strange attractor, and tends towards, or already is in, the state with a lower number of possible directions of further evolution (Devaney, 1986). To sum up, in the light of fractal organisation theory, forest integrity refers to empirical scaling relationships that are emergent features of biodiversity (see Messier et al., 2015; Simard et al., 2013; Chen, 2012; Marks-Tarlow, 2012; Raye, 2012; Willerslev and Pedersen, 2010; Gorshkov et al., 2004; Brown et al., 2002; Turcotte and Rundle, 2002; Kirilyuk, 2002; Sandywell, 1996).

General suggestions

What is sustainability in the context of forest integrity? It is maintaining scaling relationships inherent to self-organisation (see Graham, 2014; Simard et al., 2013; Rozenfeld et al., 2009; Rickles et al., 2007; Sandywell, 1996). In the presence of intransitivity, the forces driving self-organisation can be analysed with game theory (the analysis of group interaction). However, intransitivity implies that every alternative is dominated by another alternative, so no one pure strategy can be argued to be any better than another (see Ficici and Pollack, 2003; Cooter, 2000). Therefore, sustainable forest management guided by the principles of self-organisation is to be based on a collective of strategies (see Graham, 2014; Cornett and White, 2013; Schütz, 2011; Willerslev and Pedersen, 2010; Li and Bowerman, 2010; Rennolls et al., 2007; Rickles et al., 2007; Millennium Ecosystem Assessment, 2005; Ficici and Pollack, 2003; Lindenmayer and Franklin, 2002; Lorenz, 1990). The two principles of the self-organisation are nature automation, like self-regeneration of a forest, self-differentiation of a stand, self-structuration of a community, etc., and concentration on essential, such as on a protecting key response traits and ecosystem legacies that are critical in the avoidance of a biodiversity threshold, i.e., an abrupt decline in species richness, with habitat loss (see Mackey et al., 2015; Estavillo et al., 2013; Simard et al., 2013; Holt and Miller, 2011; Thompson et al., 2009; Rickles et al., 2007; Schütz, 2006; Diaci, 2006; Kotar, 2006; Kerr et al., 2002; Cody, 1985). In this context, 'A critical management target is conservation of genetic legacies for the system memory and adaptive capacity they provide' (Simard et al., 2013). Unfortunately, 'the vital requirements of clarity, simplicity and practicality do not appear to have been seriously considered in the formulation of many of the genetic criteria and indicators developed to date for the management and monitoring of forest resources' (Boshier and Amaral, 2004; *Table 2*).

How can geneticists help forest biodiversity adapt to changing ecoclimates? First, there should be an effort to complete vulnerability assessments and action plans for forest tree species. For example, in the U.S. Pacific Northwest and Southern Appalachian regions the Forest Tree Genetic Risk Assessment System (ForGRAS) was used to rank forest tree species for a number of primary risk factors: population structure, rarity, regeneration capacity, dispersal ability, habitat affinity, genetic variation, pest and pathogen threats, and climate change pressure (Erickson et al.,

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2012). Second, the conservation of biodiversity implies that the biotic verifiers, such as habitat shifts (*Table 2*), should necessarily be used by auditors and managers to derive an objective decision on the quality of the forest management under assessment. If wild organisms are extracted from their habitats and placed under artificial conditions never encountered in their natural environment, a decay of the genetic information will be manifested as an increase in genetic polymorphism of the populations and appearance of organisms with various defective properties not encountered in the wild type (Gorshkov and Makarieva, 1997; Gorshkov et al., 2004). Nevertheless, studies simulating the impact of forest exploitation, other silvicultural practices and forest fragmentation on genetic diversity are uncommon, and those that exist usually contain oversimplified representations of biological processes (Degen et al., 2004; Gorshkov et al., 2004).

An exploration of fractal connectivity behind key bioindicators and biomonitors in the avoidance of a biodiversity threshold can be suggested thus far. According to Holt and Miller (2011), bioindicators or biomonitors rely upon the complicated intricacies of ecosystems and use a representative or aggregated response to convey a dynamic picture of the condition of the environment. For instance, lichen diversity is commonly used as a general indicator of forest health and ‘ecological function’, as lichens are key primary producers with important linkages to nutrient cycling and forest food webs: high or low lichen diversity can result from certain types of air pollution, changes to forest management or stand structure, diversity of plant substrates available for colonization, favourability of forest climate, return interval of disturbances like fire, and so on (Jovan, 2008).

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Table 2. *Proposed indicators and verifiers of the maintenance of genetic diversity in sustainable forest management at interspecific, species and infraspecific levels. Sources: Déri et al. (2010), Rodriguez et al. (2009), Magura et al. (2006), Gorshkov et al. (2004), Boshier and Amaral (2004), Namkoong et al. (2002), Legendre and Legendre (1998)*

Indicator	**Biotic verifiers**	**Demographic verifiers**	**Genetic verifiers**
Levels of variation	Species' site specificity Species' site fidelity Habitat affinity index	Census number of sexually mature individuals Census number of reproducing individuals Coefficient of phenotypic variation	Number of alleles Gene diversity Genetic variation
Directional change in allele or genotype frequencies	Habitat shifts	Phenotypic shifts Age/size class shifts Environmental shifts	Genotypic frequency shifts Marker frequency shifts Genetic mean shifts
Migration among populations	Forest removal Propagule removal	Physical isolation Mating isolation Seed dispersal Pollen dispersal	Gene flow
Reproductive processes/mating system	Biotic regulation Symbiotic regulation	Parental pool size Seed germination Pollinator abundance Sexuality	Outcrossing rate Correlated mating

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MOWING INTENSITY INFLUENCES DEGREE OF CHANGES IN CARABID BEETLE ASSEMBLAGES

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Abstract. Mowing measures are frequently used as a tool for species conservation. However, mowing intensity as well as ecological characteristics of different sites may have an influence on the respective species or assemblages. Based on a BACI study design we tested if the effect of mowing on carabid assemblages is more pronounced with a higher intensity of the mowing measures, and if the results are influenced by differences in environmental characteristics between individual study sites. Based on 2650 carabid individuals belonging to 43 species we demonstrated that an intensification of mowing intensity resulted in an increased effect on the carabid coenoses. While assemblages from study sites mown once in the year were located closer to those from unmown sites, assemblages from study sites mown twice in the year were clearly separated in ordination analysis. However, some environmental variables, as shown by plant indicator values, accounted for a comparatively high amount of the variance. The study confirms that in the context of using mowing as a measure for nature conservation the mowing intensity has to be taken into account, but also variation in environmental factors.
Keywords: *Carabidae, post agricultural land, mowing measures, biological diversity, BACI*

Introduction

Since long man has influenced his surrounding nature and environment. For example, with cutting of woodland in the middle ages agricultural landscapes became dominant in Europe. Changes in agricultural practices in the recent past lead to intensification of agriculture and change in land use, which are assessed to be main drivers of loss in biological diversity (e.g. Matson et al., 1997; Watt et al., 2007; Haźi et al., 2012). Thus, retransformation of agricultural production in order to contribute to ecosystem integrity is a key challenge of the twenty-first century (Scherr and McNeely, 2008). According to Gonthier et al. (2014) conservation strategies in agriculture require a multi-scale approach including reducing local management intensity and landscape-level approaches incorporating natural or semi-natural areas.

Traditional agricultural practices, as mowing in order to produce hay, should receive special attention. Mowing has also been proposed as a surrogate for grazing (Morris, 2000). Yet, diversity patterns depend on management intensity. For example, Kitahara et al. (2000) observed a highly significant decrease in butterfly species numbers with increasing mowing intensity. Likewise, Marini et al. (2000) detected reduced orthopteran and butterfly diversity with high fertilisation and cutting frequency. Carabid beetles are known to react to management practices in grassland habitats as well (Rainio and Niemelä, 2003) and are sensitive to human-altered abiotic conditions (Koivula, 2011). For example, regarding mowing of Swiss montane meadows Grandchamp et al. (2005) demonstrated a positive influence of fertilization intensity on species richness.

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Number of cuts was positively related to the number of individuals. Mown sites in the central-eastern Alps had significantly more species than natural grasslands (Gobbi et al., 2015). Carabid beetles play an important role in agricultural systems, for example as biological control agents on agricultural pests (Humphreys and Mowat, 1994) or seed predators of weeds (Saska, 2008; Petit et al., 2014).

In 2013 a study was started following a "before-after-control-impact" (BACI) study design in order to analyse the effect of mowing measures on carabid beetles. A rather low management intensity in the second year of study (one-time mowing in the first week of July 2014) resulted in a rather weak effect of the mowing measures (Schwerk and Kitka, 2016). Hence, it remained an open question, if an intensification the mowing measures will lead to a more pronounced effect on the carabid assemblages. Moreover, differences in site characteristics independent from the mowing measures might have an additional influence on the formation of the carabid coenoses. Therefore, we increased the mowing intensity from one-time mowing to mowing twice in a third year of the study. In addition, we conducted phytosociological surveys on the study sites in order to specify site characteristics not related to the mowing measures.

The aim of the presented paper was to test (1) if the effect of mowing on carabid assemblages is more pronounced with a higher intensity of the mowing measures, and (2) if the results are influenced by differences in environmental characteristics between individual study sites.

Material and methods

Study sites and field methods

The study was carried out in 2013-2015 on post-agricultural fallow land abandoned from crop production for 21 years in 2013, located in the research area “Krzywda” in Western Poland (Dymitryszyn et al., 2013). Todays’ relief of the region is connected to the last phase of the Baltic glaciation with sand and loams covering almost the whole area. It is located in the zone of Atlantic climate with western wind dominating and a yearly rainfall of about 500-600 mm (Kucharski and Pawlaczyk, 1997; UMiG Tuczno, 2001). According to Matuszkiewicz et al. (1995) “Krzywda” is situated in the range of continental mesotrophic oak-pine mixed forest (Pino-Quercetum).

The study followed a "before-after-control-impact" (BACI) design with three treatment sites and three control sites. Thus, six study sites (B1 – B6) of 50 m x 50 m were established, three of which (B1, B4, B5) were mown with biomass removal on July, 5, in 2014 and on June, 26-27, and August, 17-18, in 2015. The remaining three study sites (B2, B3, B6) were left untreated in 2014 and 2015 (*Figure 1*).

Carabids were collected as described by Schwerk (2014a) using pitfall traps (Barber, 1931) from mid-May to mid-September. Three pitfall traps (distance 3 m) were installed in the center of each study site. The location of the traps was the same in each year of the study. Traps were glass jars topped with a funnel (upper diameter of about 10 cm) set flush with the soil surface. A roof was suspended a few cm above the funnel and ethylene glycol was used as a killing agent and preservative. In order to guarantee their proper functioning the traps were regularly controlled (about every two weeks). Determination and nomenclature of the individuals collected was carried out according to Freude et al. (2004).

At each study site an area of 6 m x 6 m was marked in order to elaborate a phytosociological survey. The surveys were elaborated in the second week of June 2015

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(before the first mowing on the treatment sites) by recording the species and describing their occurrence using the cover-abundance scale of Braun-Blanquet (1964).

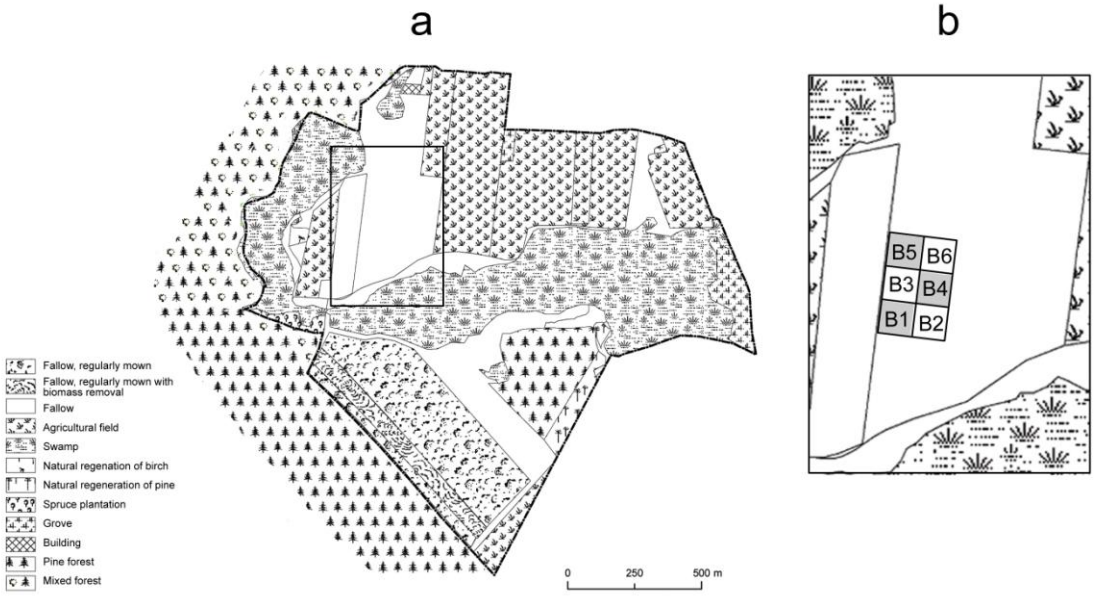

Figure 1. *Scheme of the research area "Krzywda" (a) with the location of study sites B1 – B6 (b). Study sites mown in 2014 and 2015 are indicated by grey colour (from Schwerk and Kitka, 2016)*

Statistical methods

The results of the three traps were pooled to one sample for each study site. For each species, the total number of individuals per study site and the dominance value (percentage share of the individuals of the respective species on the total number of individuals collected at the study site) were calculated.

For each phytosociological survey the values of coverage of the plant species were transformed to a value of mean percentage cover according to Braun-Blanquet (1964): + – 0.1%, 1 – 5%, 2 – 17,5%, 3 – 37,5%, 4 – 62,5%, and 5 – 87,5% (*Appendix 1*). Next, for each study site ecological indicator values of vascular plants were calculated according to Zarzycki et al. (2002). The ecological values according to Zarzycki are a modification of the method of ecological values of vascular plants according to Ellenberg (1974) adapted to the conditions of the Polish climate. Ecological indicator values for light, temperature, soil moisture, trophy (fertility), soil acidity (pH), soil granulometric composition, and organic matter content (humus) were calculated for each study site as a weighted average value of particular plant species cover values.

In order to analyse the similarities between the carabid coenoses a hierarchical cluster analysis was carried out using PAST v. 2.17c (Hammer et al., 2001; Hammer, 2012), with Euclidian distance as distance measure and agglomeration according to Ward. The dominance values of the species at the respective study sites were used. The strength of the nodes was tested by bootstrapping analysis (1999 resamplings). Bootstrap proportions (percentage of replicates where the node is still supported) of ≥70% correspond to a probability of ≥95% that the respective clade is correct (Hillis and Bull, 1993).

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We carried out gradient analyses using the CANOCO for Windows version 4.53 (ter Braak, 1987; ter Braak and Šmilauer, 2002) with the aim to obtain information about the environmental basis determining the major pattern in variation (ter Braak and Prentice, 1988). DCA was first used to select the appropriate statistical model based on the longest gradient (ter Braak and Prentice, 1988) and then principal components analysis (PCA) was carried out. PCA was done using inter-sample distances without post-transformation of species scores. Because dominance values were used, the data were not transformed. CanoDraw for Windows version 4.14 was used to create a biplot with species fit range adjusted in such a manner that the 10 species with the highest fit into the ordination space were displayed (ter Braak and Šmilauer, 2002).

In order to test the significance of the individual environmental variables and the mowing treatment (defined as nominal variable) in 2015, we carried out a redundancy analysis (RDA) with Monte Carlo permutation tests (unrestricted, 1999 permutations) first for each variable separately and then using automatic forward selection of variables (reduced model) using CANOCO for Windows version 4.53 (ter Braak and Šmilauer, 2002).

Results

During the three years of study 2650 carabid individuals belonging to 43 species were collected. Total numbers of collected individuals declined constantly during the study, whereas total numbers of species increased from 2013 to 2014 but dropped from 2014 to 2015. A decline in numbers of species and individuals in 2015 is particularly evident for the control sites (*Appendix 2*).

The calculated plant indicator values exposed differences between the individual study sites (*Table 1*). High results for all indicator values were detected on study site B2, whereas study sites B5 and B6 exhibited comparatively low results for the majority of the indicator values. Thus, the values indicated a slight north-south gradient with respect to the site characteristics. Moreover, it is striking that the results for light values are particularly high on the control sites.

Table 1. *Plant indicator values recorded for the study sites in 2015 (L, light; T, temperature; M, moisture; Tr, trophy; A, acidity; GC, granulometric composition; H, humus)*

Study site / site type	L	T	M	Tr	A	GC	H
B1 / Treatment site	3.92	3.65	3.02	3.43	3.90	3.92	2.00
B2 / Control site	5.23	4.38	3.10	3.55	4.78	4.38	2.33
B3 / Control site	4.47	3.61	2.44	2.79	3.79	3.47	1.97
B4 / Treatment site	4.20	3.64	2.86	3.45	3.98	3.63	1.95
B5 / Treatment site	4.70	3.52	2.11	2.32	3.47	3.14	2.00
B6 / Control site	4.99	3.50	2.01	2.01	3.50	3.01	2.00

The cluster analysis separated the carabid coenoses into two basic clusters, strongly supported by a bootstrapping proportion of 100% (*Figure 2*). The first cluster contained all samples of study sites mown twice in 2015, two samples of study sites, which were mown once in 2014 (B4, B5) and the sample of the control site B6 from 2015. This cluster was divided into two clusters (bootstrapping proportion of 24%), resulting in a separation of study sites B1 and B4 from 2015 (two times mown) from the remaining

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sites. The second basic cluster contained all remaining samples from not mown sites (i.e. all remaining samples from control sites and the samples from B1, B4 and B5 from 2013) and also the sample from study site B1 from 2014, which was mown one time. Within this cluster the samples from study B6 of the years 2013 and 2014 were separated from the remaining samples (bootstrapping proportion of 15%).

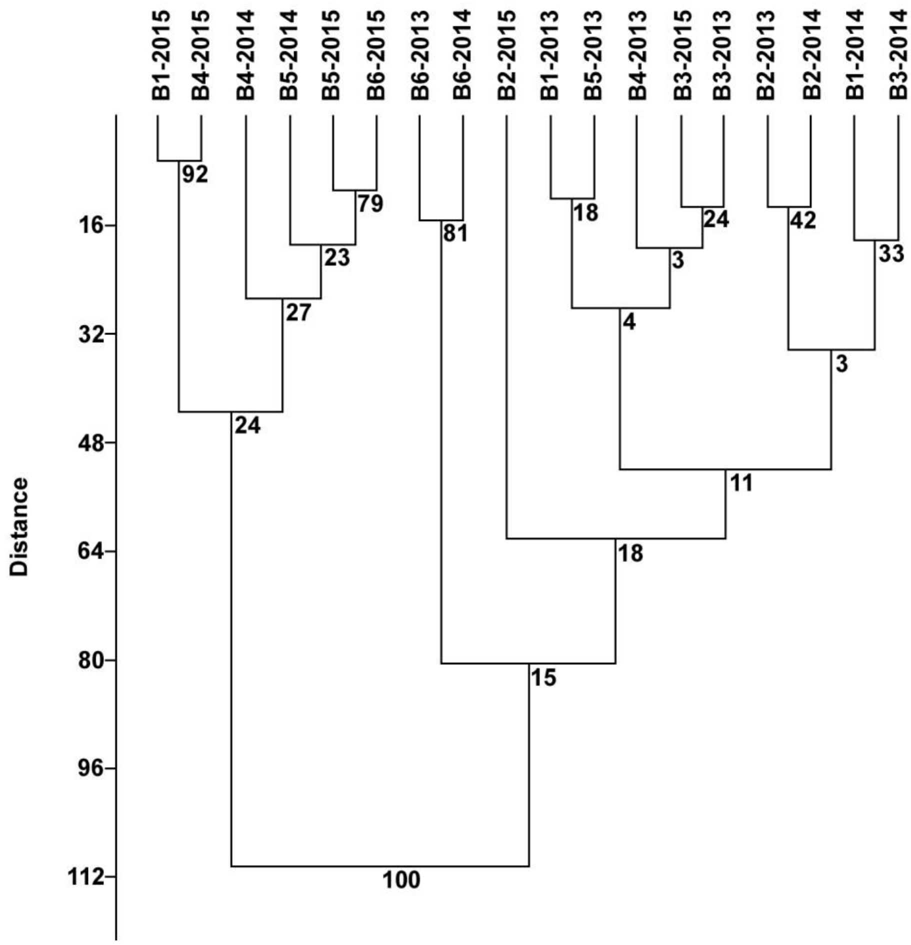

Figure 2. *Cluster analysis of the results based on Euclidian distance as distance measure and agglomeration according to Ward. Numbers indicate the percentage of replicates where each node is still supported (Hammer, 2012)*

The first and second ordination axis of the PCA (*Figure 3*) explained 39.4 % and 22.7 % of the variation in the dataset respectively. The study sites showed a distribution along the first ordination axis with respect to management intensity. Study sites two times mown in 2015 were located the most to the right side of the diagram. Two of the study sites being mown once in 2014 (B4, B5) were also somewhat shifted to the right side. However, the sample of study site B1 from 2014 (also mown one time) was located on the left side together with the majority of unmown sites, i.e. samples from treatment sites of the year 2013 and control sites. All three samples on study site B6 are located most on the upper site of the diagram along the second ordination axis. The sample of the year 2015 of this study site is also shifted quite far to the right side of the

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diagram. Among the species *Calathus fuscipes* was related to samples from study sites which were two times mown, whereas *Calathus erratus* took an intermediate position between samples from study sites two times mown and study site B6. *Amara aenea* was related to study site B6. Species related to samples from unmown study sites were *Harpalus luteicornis*, *Poecilus versicolor*, and *Pterostichus niger*.

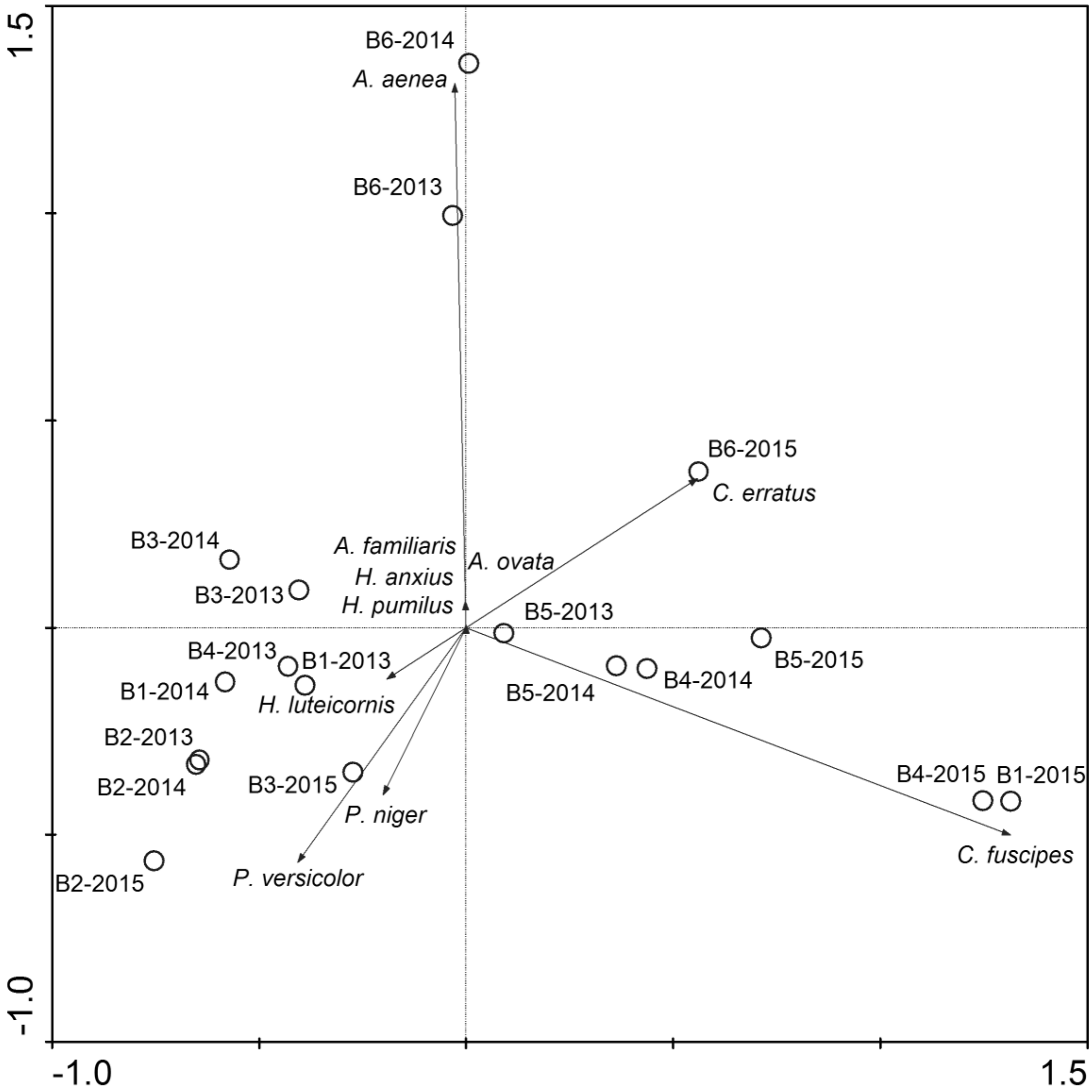

Figure 3. *Ordination plot based on principal components analysis (PCA) of the results for study sites (open circles) and species (arrows)*

Testing the environmental variables separately using Monte Carlo permutation tests revealed highest Lambda-1 values (above 40%) for humus content, temperature, light, and mowing (not mowing respectively), i.e. these variables accounted for most of the variance when considered singly (*Table 2*). However, none of them was statistically significant. When using forward selection Lambda-A values (additional variance the variable explained at the time it was included in the model) for temperature, light and the mowing measures are considerably reduced compared to the Lambda-1 values.

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Discussion

Our results indicated that the management intensification from one-time mowing (in 2014) to mowing twice (in 2015) indeed resulted in an increased effect on the carabid coenoses. The cluster analysis resulted in a strong supported separation of almost all mown sites from the unmown sites and a weaker separation of two of the sites mown twice from the sites mown only once. However, the increased effect is particularly visible in the position of the carabid samples along the first ordination axis in the PCA. In Welsh peatland strong grazing by cattle and horses resulted in a strong influence on carabid assembages compared to an only little influence due to light sheep grazing (Holmes et al., 1993). Kaltsas et al. (2013), who investigated the response of carabid beetles to grazing in Cretan shrublands, described carabids as good indicators of grazing pressure at assemblage level, rather than species-specifically. Overgrazing resulted in lower species richness, and species richness and diversity were maximal under moderate to relatively high levels of grazing. Similarly, in a study by Mayr et al. (2007) carabid beetle diversity was the highest in grasslands with medium management intensity. In the study by Grandchamp et al. (2005) the number of cuts was positively related to the number of individuals. Since in our study a decline in numbers of species and individuals was visible particularly on the control sites, it seems that mowing at least counteracted trends of declining species and individual numbers.

Table 2. *Results of Monte Carlo permutation tests of the environmental variables tested separately and using automatic forward selection of variables (reduced model). During forward selection of variables "Temperature", "Light", "Unmown", and "Trophy" were not added to the model due to collinearity. Lambda-1 – variance explained by the environmental variables separately; Lambda-A – additional variance explained when included in the model using forward selection*

Variable	Tested separately			Forward selection		
	Lambda-1	F	*p*	Lambda-A	F	*p*
Humus	0.47	3.56	0.111	0.47	3.56	0.111
Temperature	0.46	3.42	0.109	-	-	-
Light	0.44	3.16	0.058	-	-	-
Mown	0.44	3.13	0.099	0.02	0.00	1.000
Unmown	0.44	3.13	0.104	-	-	-
pH	0.39	2.55	0.117	0.12	5.71	0.176
Gran. comp.	0.29	1.67	0.218	0.15	2.17	0.243
Moisture	0.22	1.12	0.372	0.24	2.41	0.108
Trophy	0.22	1.12	0.363	-	-	-

Among the carabid beetle species *Calathus fuscipes* showed the most distinct connection to the mowing treatment. *Calathus erratus* also benefitted from the mowing measures, but was also regularly frequent on study site B6. This result is in accordance with Schwerk and Szyszko (2009) and Schwerk (2014b), who detected highest abundances of these species on regularly mown fallow grounds. *Calathus fuscipes* seems to be particularly sensitive to management intensity, since it also showed higher numbers of individuals in agricultural fields subjected to conventional tillage with ploughing compared to fields with non-inversion tillage (Kosewska et al., 2014). The special position of study site B6 in the ordination diagram as well as the grouping of B6 from 2015 with the treatment sites in the cluster analysis points to special environmental

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characteristics specific for this study site. This aspect is supported by the differences in site characteristics revealed by the indicator values elaborated for 2015. The conclusion is possible that *Calathus erratus* reacts also on some characteristic not related to mowing. Accordingly, despite in average highest abundances on the treatment sites in 2014 (one time mown), Schwerk and Kitka (2016) could not demonstrate a statistically significant reaction of *Calathus erratus* on mowing. *Harpalus luteicornis*, *Pterostichus niger*, and *Poecilus versicolor* were closer related to not mown study sites. Former studies had already shown that *Poecilus versicolor* reacts much less on mowing measures than *Calathus fuscipes* and *Calathus erratus* (Schwerk and Szyszko, 2009; Schwerk, 2014b). *Pterostichus niger* is known as species preferring forest habitats, but was also detected regularly in different field margins (Asteraki et al., 1995). This species reacted significantly on a management intensity of one-time mowing on the study sites (Schwerk and Kitka, 2016).

A significant meaning has to be attributed also to differences in environmental characteristics between the sites, as for example the special position of study site B6 in the ordination diagram indicates. Several of the variables seem to be largely replaceable by each other, as indicated by the reduced Lambda-1 values when using forward selection. In our study particularly organic content (humus) accounted for a high amount of the variance in the assemblages. The significance of this parameter has been already described in literature. Gardner et al. (1997), studying the consequences of grazing pressure on carabids on heather moorlands in northeast Scotland, detected a strong influenced of soil organic content. In Welsh peatland biotopes the nutrient status and saturation of the substrate were most important with respect to carabid assemblages (Holmes et al., 1993). Sądej et al. (2012) showed a positive relation between soil organic matter and carabid species number in experimental agricultural fields in northern Poland.

According to Digweed et al. (1995) distances of 50 m should provide adequate statistical independence between traps. Yet, it cannot be excluded that the one-time mowing in 2014 on the treatment sites contributed to some degree to the more pronounced differences on these sites in 2015. However, since the effect of the mowing measures in 2014 was quite weak, we assess an influence on the results in 2015 as rather insignificant. It is reasonable to assume that the observed differences in 2015 were caused by the increased mowing intensity causing a more continuous effect on the carabids due to an earlier first mowing compared to 2014 and the additional later second mowing, which may have provoked some species to conduct linear direct walking behaviour in order to escape from unfavorable habitats (e.g. Baars, 1979; Riecken and Raths, 1996) and other species to enter the treatment sites.

Our study confirms that in the context of using mowing as a measure for nature conservation the mowing intensity has to be taken into account, since it appears that certain species can be facilitated by modifying mowing intensity. Additionally, the impact of environmental factors and their spatial variation has to be considered. In this regard attention should be paid to soil organic content. However, time of mowing is of importance too, for example delayed mowing may have a different effect on individual species or taxonomic groups (e.g. Humbert et al., 2012).

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APPENDIX

Appendix 1. *Mean percentage cover according to Braun-Blanquet (1964) of the recorded species on the study sites in 2015*

Species	B1-2015	B2-2015	B3-2015	B4-2015	B5-2-15	B6-2015
Herbaceous layer						
Achillea millefolium L.		5.0	5.0	5.0		
Agrostis capillaris L.	5.0	5.0		17.5	17.5	
Anthemis arvensis L.	0.1					
Anthoxanthum odoratum L.				5.0	0.1	0.1
Armeria elongata (Hoffm.) W. D. J. Koch			0.1		0.1	
Arrhenatherum elatius (L.) P. Beauv.	37.5	17.5	17.5	17.5	0.1	0.1
Artemisia campestris L.		0.1				0.1
Artemisia vulgaris L.	0.1	5.0	0.1	0.1		
Bromus sp.				0.1		
Calamagrostis epigejos Roth.					0.1	
Cardaminopsis arenosa (L.) Hayek	0.1	0.1		0.1		
Carlina vulgaris L.	0.1					
Cerastium holosteoides Fr. em. Hyl.	0.1		0.1		0.1	
Dactylis glomerata L.	0,1					
Daucus carota L.	5.0			5.0		
Deschampsia caespitosa (L.) P. Beauv.	5.0			0.1		
Deschampsia flexuosa (L.) Trin.	5.0	17.5		5.0	0.1	0.1
Dianthus deltoides L.			0.1		5.0	
Festuca ovina L.		17.5	5.0			0.1
Galium mollugo L.	0.1		5.0	5.0		
Galium verum L.	5.0	17.5	0.1	17.5	0.1	
Gnaphalium sylvaticum L.	0.1					
Helichrysum arenarium (L.) Moench	0.1		0.1			0.1
Hieracium laevigatum Willd.	0.1					
Hieracium pilosella L.	0.1	5.0	37.5	0.1	62.5	87.5
Holcus lanatus L.	5,0			17.5	0.1	
Hypochoeris radicata L.					0.1	
Leontodon autumnalis L.				0.1		0.1
Phleum pratense L.			0.1	5.0		
Pimpinella saxifraga L.			0.1		0.1	
Pinus sylvestris L.						0.1
Polygonum amphibium L.			0.1			
Potentilla argentea L.	0.1	0.1				
Rumex thyrsiflorus Fingerh.		0.1	0.1		0.1	
Scleranthus annuus L.					0.1	
Senecio jacobaea L.	0.1		0.1	0.1		
Tragopogon pratensis L.				0.1		
Veronica officinalis L.	17.5		5.0			
Vicia angustifolia L.	0.1		0.1	0.1		
Vicia hirsuta (L.) S. F. Gray	5.0		0.1			0.1
Vicia villosa Roth.	0.1		5.0	0.1		
Viola arvensis Murray	0.1	0.1	0.1			
Moss layer						
Polytrichum juniperinum Hedw.			5.0		0.1	5.0
Pleurozium schreberi (Willd.) Mitten.	5.0	5.0				

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Appendix 2. *Numbers of individuals of the recorded species on the study sites in 2013, 2014 and 2015*

Species	**B1-2013**	**B2-2013**	**B3-2013**	**B4-2013**	**B5-2013**	**B6-2013**	**B1-2014**	**B2-2014**	**B3-2014**	**B4-2014**	**B5-2014**	**B6-2014**	**B1-2015**	**B2-2015**	**B3-2015**	**B4-2015**	**B5-2015**	**B6-2015**
Agonum gracilipes (Duftschmid, 1812)			1															
Amara aenea (De Geer, 1774)	1	1	8		6	21		2	9		4	110					2	7
Amara bifrons (Gyllenhal, 1810)								1			1	6						
Amara communis (Panzer, 1797)		1	1	2	1	1		2	1	1	16	18						3
Amara convexior Stephens, 1828	6	12	23	19	5		34	11	16	7	5	11		2	3	9	4	
Amara curta Dejean, 1828				1														
Amara familiaris (Duftschmid, 1812)	1					1						6						
Amara lunicollis Schiödte, 1837	11	8	2	16	2	2	19	19	2		3		1	1		1		
Amara ovata (Fabricius, 1792)												1						
Amara plebeja (Gyllenhal, 1810)	2	4		3		1	3	3			2		1					
Amara spreta Dejean, 1831								1										
Amara tibialis (Paykull, 1798)												3				1	1	
Anisodactylus nemorivagus (Duftschmid, 1812)							1											
Calathus cinctus Motschulsky, 1850																	3	
Calathus erratus (C. R. Sahlberg, 1827)	11	1	9	18	17	14	7	1	1	35	12	37	15			38	19	13
Calathus fuscipes (Goeze, 1777)	1		5	2	17	1		2		25	28	10	40		4	112	27	12
Calathus melanocephalus (Linné, 1758)	10	4	4	5	1	5	2	5			4	9	2	3		13	12	12
Carabus granulatus Linné, 1758					1						1							
Cychrus caraboides (Linné, 1758)								1										
Cymindis angularis Gyllenhal, 1810		1	1		1							2						
Harpalus affinis (Schrank, 1781)											1				1			1
Harpalus anxius (Duftschmid, 1812)												1						
Harpalus griseus (Panzer, 1796)						2												
Harpalus latus (Linné, 1758)	4	4		11	2		4		5	9	2	1			1	15		
Harpalus luteicornis (Duftschmid, 1812)	1	15	3	8	3	2	5	12	8	10	1			6	2	3		2
Harpalus pumilus Sturm, 1818												1						
Harpalus rubripes (Duftschmid, 1812)	30	18	34	28	26	14	21	13	5	4	10	17	6	8	7	9	7	6
Harpalus rufipalpis Sturm, 1818		1																
Harpalus rufipes (De Geer, 1774)	41	42	25	49	30	12	15	9	7	25	30	24	9	8	8	22	30	16

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Harpalus signaticornis (Duftschmid, 1812)														1				
Harpalus smaragdinus (Duftschmid, 1812)	2																	
Harpalus tardus (Panzer, 1796)	12	61	19	49	3	3	6	31	7	21	8	18	2	6	5	7		2
Panagaeus bipustulatus (Fabricius, 1775)							1		1									
Poecilus cupreus (Linné, 1758)	1																1	
Poecilus lepidus (Leske, 1785)	1	1		1	3	1					1				1		4	1
Poecilus versicolor (Sturm, 1824)	23	12	8	7	10	1	15	19	2	6	6	3	4	28	4	10	2	
Pterostichus melanarius (Illiger, 1798)	6		2	3	12	1	1	1			5		3	1		1		
Pterostichus niger (Schaller, 1783)	17	46	5	13	15		5	25	12	4	11		7	4	5	19	5	
Pterostichus oblongopunctatus (Fabricius, 1787)	1		1		1													
Syntomus truncatellus (Linné, 1761)							1										1	
Synuchus vivalis (Illiger, 1798)					2		1				2						2	
Trechus obtusus Erichson, 1837																	1	
Zabrus tenebrioides (Goeze, 1777)					2													
Individuals	182	232	151	235	160	82	141	158	76	147	153	278	90	68	41	260	121	75
Species	20	17	17	17	21	16	17	18	13	11	21	18	11	11	11	14	16	11
Individuals (total for the year)	1042						953						655					
Species (total for the year)	29						32						25					

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EXPERIMENTAL STUDY ON CRYOGEN INJECTION INTO WATER

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Abstract. Explosive boiling is a hyperactive boiling phenomenon, which is characterized by transient high heat flux. There are still few researches on the experimental comparative analysis on explosive boiling inducing by different cryogen liquid injection into water. In this paper, to advance our understanding of LNG (Liquefied Natural Gas), LN_2 (Liquefied Nitrogen) and LC_3H_8 (Liquefied Propane) injection into water process, a visualization experimental system is built. Visualized results show that LNG and LN_2 injection processes undergo a similar boiling, that is explosive boiling, which is characterized by bubbles cloud that strengthens heat transfer rate. LC_3H_8 injection into water triggers subcooled flow boiling. There is no significant breaking on the liquid cryogen column and without bubbles cloud. The maximum heat transferring flux can be over 1.9 MW/m^2 in the condition of LNG injection into unconfined water and under the pressure of 7 bars. In order to investigate the determinant factors for explosive boiling occurring, instability of Rayleigh-Taylor, Kelvin-Helmholtz, Weber number and Marangoni convection are analyzed and used to explain the differences of maximum pressure and its occurring time in different experimental conditions such as injection depth into water, injection pressure, water temperature and injecting fluid. As a result, it is reasonable to conclude that if the relative velocity between cryogen and surrounding fluid is high enough, the breaking of liquid column or droplets will be defined by the Kelvin-Helmholtz instability and Weber number. And in subcooling mixture or pure substance injection process, the periphery of the vapor film near the head of column is considerably stronger than other regions in the effect of Marangoni convection at the beginning of column floating upwards or the ending of column going downwards. All of these can be seen as the key factors to improve heat transfer capacity and trigger explosive boiling.
Keywords: c*ryogen; explosive boiling; experimental study; instability; Malangoni effect; bubble behaviors*

Introduction

Explosive boiling is a hyperactive boiling phenomenon, which is characterized by transient high heat flux. It is involved in many industry fields such as nuclear power, laser printing, microelectronic, fuel-coolant interactions (FCIs) and liquefied natural gas transportation. In liquefied natural gas transportation process, when discharging liquefied natural gas into water, direct contact heat exchange between liquefied natural gas and water with different temperatures about 180K would occur. Under some certain conditions, liquefied natural gas vaporization rate will rapidly increase and the heat flux is higher than the critical heat flux (CHF). However, such rapid heat and mass transfer process would not always take place. So far, the determinants of this phenomenon have not been clearly confirmed.

Some experimental studies on discharging cryogen on water have been reported since 1971. Nakanishi and Reid (1971) conducted a series experiments on four different phases liquefied natural gas discharging on water. Experiments showed superheating occurred until homogeneous nucleation arises or until heterogeneous nucleation takes place on

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small active impurities. If the substrate was chemically like the cryogen spilled and the interfacial liquid had a low freezing point, then the explosive boiling may occur. Garland and Atinson (1971) found a similar conclusion as Nakanishi and Reid. They thought the composition of liquefied natural gas was important in noticing explosive boiling and that the presence of a hydrocarbon film on water increased the probability of explosive boiling occurrences. Enger (1972) reported a series tests of Shell, which conducted experiments on different composition liquefied natural gas discharging on hot water. The experiments showed that in environmental temperature, when methane content in liquefied natural gas was not less than 40% along with a few mole present n-butane, the explosive boiling may occur. But from Koopman et al. (1982) and Goldwire et al. (1983) reports, the explosive boiling may occur when methane content in liquefied natural gas as high as 90%. Dahlsveen et al. (2001) conducted a process for liquefied natural gas injection. With changing composition of liquefied natural gas, experiments showed a much thicker vapor blanket and larger jet spreading angle (Dahlsveen et al., 2001; Khan and Noor, 2017)). Wen et al. (2006) conducted a series experiment of liquid nitrogen injection into water. With synchronized pressure and temperature measurement, heat transfer and pressurization rates had been establishing for application to a cryogenic engine. Pressurization rates of up to 5 bar/s were recorded, and heat transfer coefficients approximated for the latent heat transfer with assumed surface area were comparable to values found in other works on boiling heat transfer over very rough surfaces (Wen et al., 2006). Clarke et al. (2010) conducted a visualization study of liquid nitrogen injection into water with synchronized pressure and temperature measurement, to obtain insight into this phenomenon. It revealed a four-stage evolution of liquid nitrogen jet structure upon injection into water, with a thick vapor blanket forming around a liquid nitrogen core and break-up brought on predominantly through impact with the vessel wall. Maximum pressurization rate in excess of 350 bar/s were recorded and found to vary proportionally with injection pressure (Clarke et al., 2010; Anees et al., 2017).

Although previous studies have provided the experiences studies for explosive boiling, there are still few researches on the experimental comparative analysis on explosive boiling inducing by different cryogen liquid injection into water. In this paper, to advance our understanding of LNG (Liquefied Natural Gas), LN_2 (Liquefied Nitrogen) and LC_3H_8 (Liquefied Propane) injection into water process, a visualization experimental system is built. Pressure profiles are obtained and synchronized with images for obtaining the heat transferring flux. Base on the instability theories, the effect of the material properties of cryogen liquid, water temperature and injection depth into water are investigated, which is in attempt to find the key factors caused explosive boiling in the process. The bubble behaviors are also analyzed and found some characteristics to explain the extremely rapid heat transferring rate of explosive boiling (Deng et al., 2017).

Experimental apparatus

The experimental rig (*Fig. 1(a)-(b)*) consists of a condensation system, a mixer tank, an injector, a visualized pressure tank (*Fig. 1(c)*), and a data acquisition system (Zhang et al., 2015, 2016).

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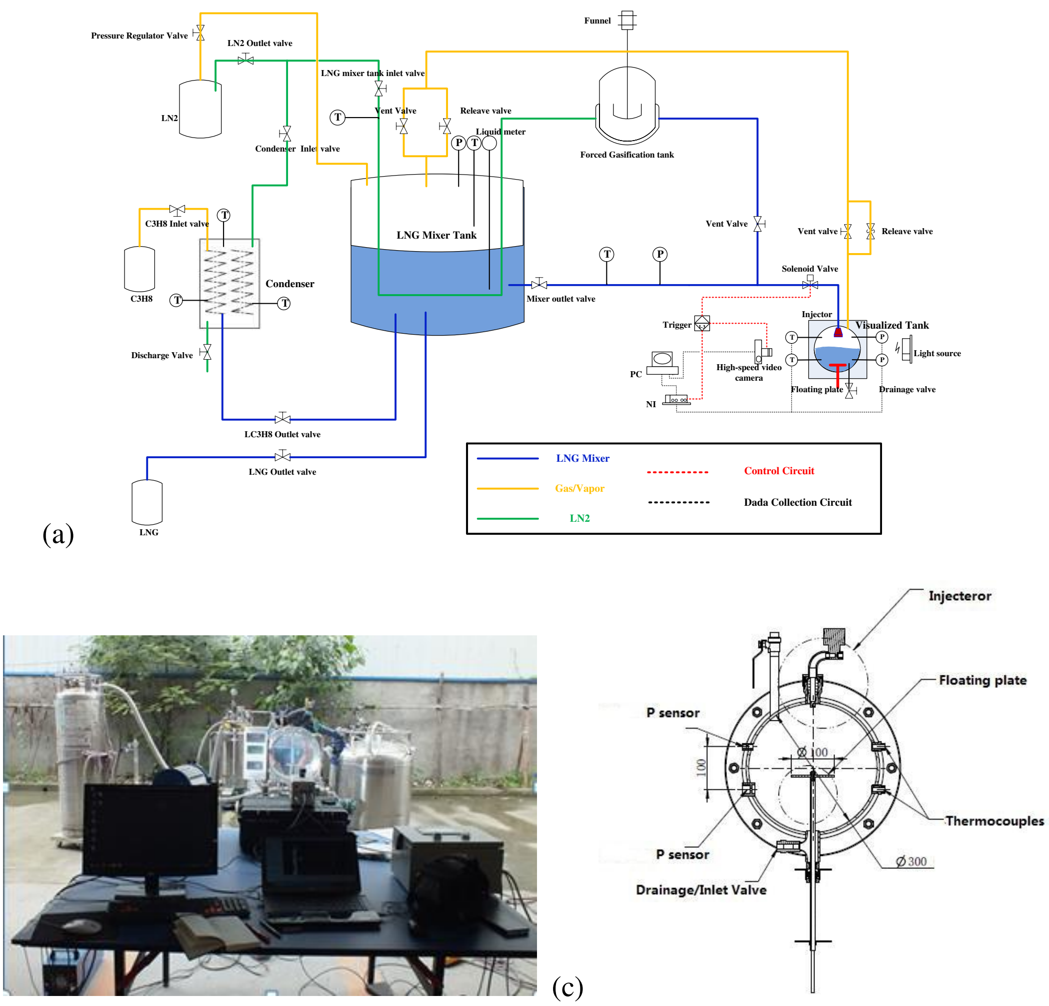

Figure 1. *LNG injection setup and apparatus: (a) schematic of experimental rig; (b) picture of apparatus; (c) positions of all sensors in tank.*

LN_2 and LNG are separately delivered through insulated pipe and outlet valve from a controllable pressure Dewar. Out of necessity, LNG injection must occur below its bubbling point, which can be conducted in the LNG mixer tank by LN_2. After the LN_2 delivered through LNG mixer tank, the temperature of LNG in mixer tank can be maintained at 100K(±5K) , that is ~10K below the LNG bubbling point. The pressure of LNG mixer tank equals to injection pressure, which is controlled by manually adjusting the pressure regular valve on LN_2 Dewar. Because the propane gas is storage in the gaseous phase, before injecting, it must be condensed into liquefied propane, which can be conducted in a similar way like LNG cooling process. In the LN_2 injection process, the system must be improved and the LN_2 Dewar outlet valve to be directly combined with the mixer outlet valve. Where after, the cryogen liquid is delivered to an ASCO intrinsically safe type cryogenic solenoid valve, which controls the opening of the injector. The injector is made of a 25mm length of steel pipe with an inside diameter of 5 mm. To prevent heat leakage, the all cryogenic liquid pipes are made of double insulated vacuum steel pipe, and before experiments, the pipes are precooled by LN_2 and the time is not less than 10 min.

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The visualized pressure tank is a stainless cylinder of diameter 300 mm and depth 150mm with Perspex windows clamped onto each end. The safety valve and vent valve are fitted on the tank to prevent pressurization above 40 bars and can vent the vapor respectively. Two T-type sheathed thermocouples of 10mm in diameter, 140mm in length, with accuracy of 0.5% FS and two high frequency dynamic pressure sensors of 4mm in diameter, 10mm in length, with accuracy of 0.5% FS with minimum response time of 4μs are used to measure the temperatures and pressure within the tank. The positions of all sensors are illustrated in *Fig. 1(c)*.

The boiling process of LNG into water is recorded by a high-speed camera (Svsi Gigaview) with the frame rate of 532~17045 frames per second. In this series experiments, the frame rate is 1000 frames per second, and frame size is 640×480 pixels. A 575 W quartz metal halide light source is added to improve the qualities of the frames. The camera and the image acquisition software are triggered by the leading edge of a 5 V pulse generated within PLC (Programmable Logic Controller) hardware, programmed and controlled by the operator. The image acquisition software comes with the camera and can set the image acquisition rate, image pixel and exposure time. The same pulse triggers the National Instruments Data-Acquisition hardware and LabVIEW to acquire and store both pressure and temperature data, at a rate of 10,000 samples per second. Another 5V pulse is generated after 100 ms within the PLC, which is used to trigger the ASCO solenoid valve opening. The opening time is dependent on the trigger pulse width, which can be set manually within the PLC.

Experimental results

By controlling the LN_2 Dewar outlet pressure and outflow, the different injection pressures and thermodynamic states of cryogen can be adjusted. A number of cryogen injection experiments are conducted, of LNG, LN_2 and LC_3H_8, into water of different temperature and depth. The parameters for experiments are shown in *Table 1*. The LNG component concentration is provided by the LNG supplying company. By adjusting the location of floating plate under water, the cryogen injection into water depth can be controlled. Injecting during time can be controlled by the power time of ASCO solenoid valve.

Table 1. *Experiment run parameters*

Run	Component Concentration CH_4:C_2H_6:C_3H_8:OS (v/v %)	Water Temperature (K)	Floating Plate Location under Water (mm)	Injection Pressure (Bar)	Injection During time (ms)	Max. Pressure (Bar)	Max. Pressure Occurring Time after Injection (ms)
1	97 : 2 : 0.5 : 0.5	290	210	7	200	3.01	6553
2	97 : 2 : 0.5 : 0.5	290	140	7	200	1.61	6284
3	97 : 2 : 0.5 : 0.5	290	210	5	200	1.64	5558
4	97 : 2 : 0.5 : 0.5	308	140	7	200	1.00	6442
5	C_3H_8: 100%	303	200	4	300	0.23	12465
6	OS: 100%N_2	290	210	7	200	2.44	5946

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Discussion

Heat transferring flux

As *Table 1* shown, the maximum pressure occurs in run 1, which injection pressure is one of the highest, water temperature is one of the lowest and floating plate location under water is bottommost. The pressure of the visualized pressure tank is shown in *Fig. 2(a)* over a 10 s timescale and additionally in *Fig. 2(b)* for the maximum pressure occurring time over 100 ms timescale (from 5400 ms to 5499 ms after injection). In *Fig. 2(a)*, the pressure data is recorded by the high frequency dynamic pressure sensors of 10 mm in length, before trigger activating 100 ms. In *Fig. 2(b)*, the pressurization is extremely rapid at ~300 bar/s. Using TNT equivalent model can simplify the process so that the heat transfer coefficient can be obtained. The following expressions relating the maximum pressure and the heat transfer flux (Zhang et al., 2015).

$$W_{TNT} = k\frac{aW_{LNG}Q_{LNG}}{Q_{TNT}} \quad \text{(Eq.1)}$$

$$\Delta P = P_0\left(3.9Z^{-1.82} + 0.5Z^{-1}\right) \quad \text{(Eq.2)}$$

$$Z = R\cdot W_{TNT}^{-1/3} \quad \text{(Eq.3)}$$

$$H_{RPT} = L_V\cdot W_{LNG}/\left(S\cdot t_d\right) \quad \text{(Eq.4)}$$

Where W_{TNT} is TNT equivalent, kg; W_{LNG} is LNG mass which is fragmented, kg; k is surface burst coefficient; a is equivalent coefficient; Q_{LNG} is bursting heat, J; Q_{TNT} is TNT bursting heat, J; ΔP is maximum pressure value, Pa; R is distance from pressure sensor, m; H_{RPT} is heat transfer flux, W/m^2; L_V is latent heat of LNG, J/kg; t_d is the duration time of pressure increasing, s. It is ~10 ms obtained from the *Fig. 2*. Combining Eqs. (1)-(4), the maximum heat transferring flux can be over 1.9 MW/m^2. It is ~7.8 times bigger than the theoretical limit of heat transferring flux of the pure liquefied methane nucleate boiling (0.244 MW/m^2) (Nail, 1999).

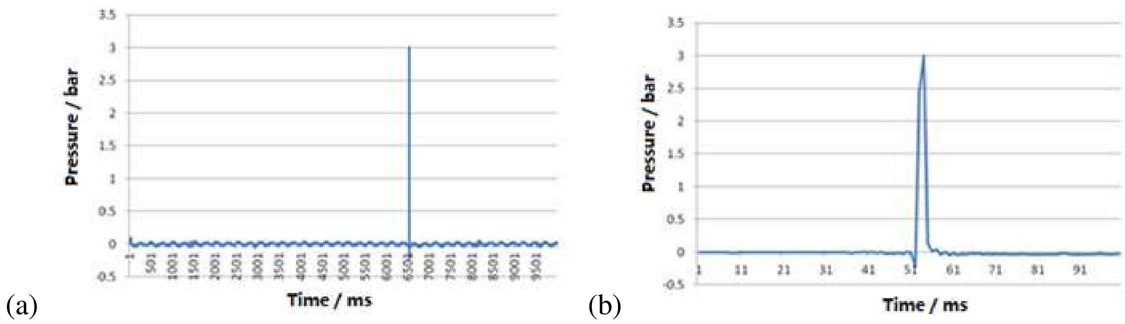

Figure 2. *Pressure curve correspondent to run 1 using the P sensor under water: (a) the time is from 0 ms to 10000 ms after injection; (b) the time is from 5400 ms to 5499 ms after injection).*

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Theoretical analysis about instability

As cryogen injecting into water, the interface between cryogen and water would be with wave. If disturbances of all wavelengths are present, there will be some disturbances at small wave number and long wavelength that will amplify and cause the interface to be unstable. There are four main instability theories can be used to explain the explosive boiling mechanism of cryogen injection into water.

Rayleigh-Taylor instability (Van, 1992; Byoung et al., 2015; Awasthi, 2013)

It is the instability of an interface between two fluids of different densities which occurs when the lighter fluid is pushing the heavier fluid. If the energy of interface tension is higher than the sum of kinetic energy of fluctuations and potential energy, the interface will be with a stable vapor film. Otherwise, the interface will be unstable and the vapor film will be broken because of the Rayleigh-Taylor instability. It takes more effect on the leading end than the side of liquid column. The most dangerous wavelength is given by:

$$\lambda_D = 2\pi\sqrt{\frac{3\sigma}{|\rho_{cl} - \rho_w|g}} \quad \text{(Eq.5)}$$

Where λ_D is the most dangerous wavelength, m; ρ_{cl} is liquid cryogens density, kg/m^3; ρ_w is circumstance fluid density, kg/m^3; σ is the interfacial tension, N/m; g is the gravitational acceleration, m/s^2.

Kelvin-Helmholtz instability (Van, 1992; Awasthi et al., 2012; Hyun and Junseok 2015)

When there is a sufficiently large velocity difference across a small amplitude perturbed interface between two fluids, the interface is unstable. This interfacial instability is known as the Kelvin-Helmholtz instability. The instability occurs when the destabilizing effect of shear across the interface overcomes the stabilizing effect of gravity and/or surface tension. It takes obvious effect on the side of liquid column. If a specific disturbance wavelength is imposed on the system, the interface will be unstable for:

$$\left|\overline{u_{cl}} - \overline{u_w}\right| > u_c \quad \text{(Eq.6)}$$

$$u_c = \sqrt{\frac{2\pi\sigma(\rho_{cl} + \rho_w)}{\lambda_D \rho_{cl}\rho_w} + \frac{g\lambda_D(\rho_{cl} - \rho_w)(\rho_{cl} + \rho_w)}{2\pi\rho_{cl}\rho_w}} \quad \text{(Eq.7)}$$

If g is set to zero, we get the dispersion relation for a vertical interface, which implies that:

$$u_c = \sqrt{\frac{2\pi\sigma(\rho_{cl} + \rho_w)}{\lambda_D \rho_{cl}\rho_w}} \quad \text{(Eq.8)}$$

The most dangerous wavelength is given by:

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$$\lambda_D = \frac{2\pi\sigma(\rho_{cl} + \rho_w)}{u_c^2 \rho_{cl} \rho_w} \quad \text{(Eq.9)}$$

Where u_c is critical velocity, m/s; u_{cl} is liquid cryogens velocity, m/s; u_w is circumstance fluid velocity, m/s.

Weber number (Van, 1992)

As cryogen injecting into water, the velocity difference between the two fluids will produce tangential stress on the interface. If the tangential stress is bigger than the surface tension, the cryogen column will be broken into liquid droplets. The minimum diameter of stable liquid droplets is dependent on the magnitude of shear stress, which will be increased with the velocity difference increasing. The minimum characteristic length of stable liquid column or droplets is given by:

$$L = \frac{We_c \sigma}{\rho_w \left(\overline{u_{cl}} - \overline{u_w}\right)^2} \quad \text{(Eq.10)}$$

$$We_c = \frac{8}{f} \quad \text{(Eq.11)}$$

Where L is minimum characteristic length of stable liquid column or droplets, m; We_c is critical Weber number; f is friction coefficient, f=0.44 when $500<Re<10^5$.

Marangoni effect (Tang, Zhu, and Sun, 2013)

Marangoni effect is caused by the heterogeneity of the surface tension near interface of two fluids with different temperature. In subcooled flow boiling, the surface tension gradient along two fluids contact interface is raised from the temperature difference in the vapor film around the liquid cryogen column, which leads to the tangential stresses of τ_{cl} and τ_{cv} on liquid cryogen side and vapor cryogen side at the interface respectively. Due to reaction of the stresses of τ_{cl} and τ_{cv}, the liquid and vapor phase of cryogen were accelerated and the Marangoni effect formed near the interface. *Fig. 3* shows a schematic diagram to describe the Marangoni effect near a smooth and stable vapor film caused by temperature gradient and the force balance in tangential direction on the cryogen surface. Under steady state condition, the balance of forces at the interface is:

$$\tau_{cl} + \tau_{cv} = \frac{d\sigma}{dT} \Delta T / \delta \quad \text{(Eq.12)}$$

Moreover, the shear stress on vapor side at the interface τ_{cv} can be neglected since the most gases viscosity is much smaller. Therefore, the simplification of (Eq.12) is:

$$\tau_{cl} = \frac{d\sigma}{dT} \Delta T / \delta \quad \text{(Eq.13)}$$

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Strength of Marangoni effect can be characterized by the Marangoni number

$$Ma = \left| \tau_{cl} \frac{\delta^2}{\alpha_{cl} \mu_{cl}} \right| \quad \text{(Eq.14)}$$

Where τ_{cl} is shear stress on liquid side, N/m^2; τ_{cv} is shear stress on vapor side, N/m^2; δ is the characteristic length of interface, m; α_{cl} is thermal diffusivity of cryogen liquid, m^2/s; μ_{cl} is the dynamic viscosity of cryogen liquid, kg/s•m.

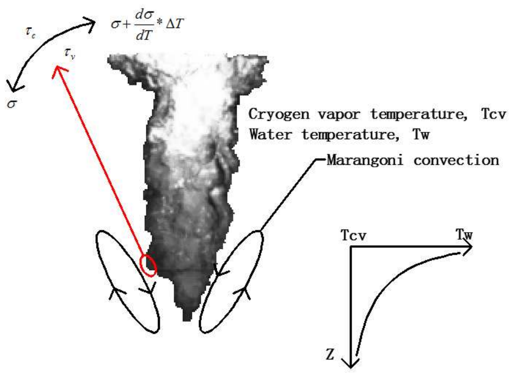

Figure 3. *Schematic diagram of Marangoni convection near the vapor film (The original image is 3d in Fig. 5)*

Effect of injection depth into water

Comparing run 1 and run 2, the maximum pressure of run 2 decreases ~46.5%, and the time of maximum pressure occurs ~270ms ahead. Besides the location of floating plate, the other parameters of experiments are similar. As a result of that, the mainly reason for the differences between the two runs is the depth of injection into water. According to the Kelvin-Helmholtz instability and critical Weber number theory, as (Eq.6) is satisfied, the interface between cryogen and water will become unstable and the liquid column of cryogen will be broken into small droplets, which diameter is equal to the λ_D or L is separately calculated by (Eq.9) and (Eq.10). The effective heat transferring area is remarkably increasing. But if the liquid column of cryogen impinges the floating plate, $\left|\overline{u_{cl}} - \overline{u_w}\right|$ will be reduced to zero, liquid column of cryogen stop breaking into droplets in the effect of instability. But the collision process will become complicated. The droplets of cryogen may be undergone two contradicting processes that are coalescence and breakup. The maximum pressure of run 2 is lower than run 1, it implies that the coalescence effect in the collision process has an obvious effect, that can decrease the heat transferring capacity and boiling rate. From another point of view, because the injection depth into water is unconfined, the breakup during time of liquid droplets or column in run 1 is longer than run 2 which induces the maximum pressure occurring time being aback.

As a result, as cryogen releasing or injecting into water, the maximum pressure or boiling rate will be reduced by confining the water depth, which can be used to limit explosive boiling occurring.

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Effect of injection pressure

Comparing run 1 and run 3, the $\overline{u_{cl}}$ in run 3 is smaller than run 1, as a result, the maximum pressure of run 3 is ~45.5% smaller than run 1. It implies the heat transfer capacity and boiling rate of run 3 are both smaller than run 1. There are two main reasons for the difference of the maximum pressure. Firstly, in the effect of Kelvin-Helmholtz instability, as (Eq.6) is satisfied, the liquid column of cryogen will breakup into droplets, and the mean diameter of droplets is equal to the most dangerous wavelength λ_D, which will be reduced with $\left|\overline{u_{cl}} - \overline{u_w}\right|$ increasing. Secondly, basing on the Weber number theory, as $\left|\overline{u_{cl}} - \overline{u_w}\right|$ increasing, the tangential stress on the interface will increase, which will decrease the minimum characteristic length L that means the liquid droplets will break into much smaller droplets. The heat transferring area will dramatically increase, which implies both boiling rate and heat transferring capacity will substantial increase.

Furthermore, the occurring time of maximum pressure is ~1000ms earlier than run 1. It means the breakup duration time of liquid column and droplets of cryogen in run 3 is shorter than run 1. In an another word, the time of liquid column and droplets stopping breaking into smaller diameter droplets in run 3 is earlier than run 1.

In conclusion, the injection pressure is related to the boiling rate. As the injection pressure increasing, the maximum pressure will increase and its occurring time will be advanced, which will increase the probability of explosive boiling.

Effect of water temperature

Comparing run 2 and run 4, the water temperature of run 4 is higher than run 2, as a result of that the maximum pressure of run 4 is ~37.8% smaller than run 2. *Fig. 4a* shows the circumstance fluid is vapor film, whose temperature is equal to the water temperature of run 2. There is a hypothesis the water temperature is low enough that there is no evaporation occurring, so the circumstance fluid is water as the *Fig. 4b* showing. Both the most dangerous wavelengths in Rayleigh-Taylor instability in *Fig. 4a-b* are approximately equal. But the most dangerous wavelengths in Kelvin-Helmholtz instability and the minimum characteristic lengths in Weber Number theory in *Fig. 4a-b* varies greatly. It implies, as the concentration of vapor in the interface between cryogen and water decreasing, the most dangerous wavelengths in Kelvin-Helmholtz instability and the minimum characteristic lengths in Weber Number theory dramatically decreasing, which intensifies the cryogen column and droplets breaking up into much smaller droplets and promotes higher heat transferring rate and boiling intensity.

Furthermore, the maximum pressure occurring time of run 4 is a little bit later than run 2. That is because, as the water temperature increasing, the initial film boiling rate and vapor film thickness in run 4 are separately higher than run 2, which will decrease relative velocity and the instability effects of the interface, as a result of that it needs much more time to trigger cryogenic column breaking into droplets and dramatically decreasing heat transferring capacity.

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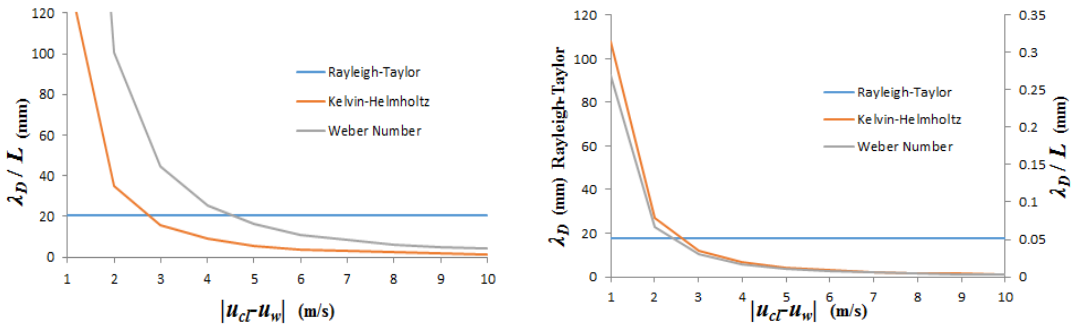

(a) Circumstance fluid is vapor of cryogen *(b) Circumstance fluid is water*

Figure 4. *Most dangerous wavelength in Rayleigh-Taylor instability and Kelvin-Helmholtz instability, and minimum characteristic length in Weber Number Theory*

As a result, if the water temperature is increasing, the instability of Kelvin-Helmholtz and Weber number will be reduced. The diameter of droplets breaking from column will be increased. The effective area of heat transferring will be considerably decreased, which will reduce the heat transferring capacity and have less opportunity to trigger explosive boiling.

Base on the thermodynamics theory, if the temperature of water is higher than Leidenfrost temperature of cryogen (Van, 1992), there will be a film boiling. The Leidenfrost temperature for methane, ethane, propane and nitrogen are 161K, 249K, 312K and 126K, respectively. And only if the water temperature is equal or slightly greater than the superheat limit temperature of cryogen ($T_{superheat} < T_{water} < 1.1T_{superheat}$), the explosive boiling will be triggered (Luketa-hanlin, 2006; Ashraf et al., 2017). The superheat limit temperature for methane, ethane, propane and nitrogen are 168K, 269K, 326K and 110K, respectively. The superheat limit temperature of hydrocarbon mixtures is approximately the mole fraction average of the superheat limit temperatures of the components (Porteous and Blander, 1975). But the water temperature is not the only factor for triggering explosive boiling. Because, in run 1, run 2 and run 3, it has the same condition of water, the maximum pressures have great differences. There should be some other factors for triggering explosive boiling, such as injection pressure, injection depth into water and working substances (Zhang et al., 2016).

Effect of injecting cryogen

From *Table 1*, the run 1, 5 and 6 are separately used LNG, LC_3H_8 and LN_2. It shows that the maximum pressure of run 1 is ~18.9% bigger than run 6, and ~13 times of run 5. Furthermore, the maximum pressure occurring time of run 6 is earlier than run 1, and much earlier than run 5. There are three main reasons.

(1) LNG is mixture. In the process of LNG injecting, the LNG column will be heated by surrounding water and some of it will boil into vapor phase. As a result of that, liquid LNG components will change and bubble point changes correspondingly. On the effect of Marangoni effect, the surface tension gradient along two fluids contact interface is raised from the bubble point difference in the vapor film around the liquid cryogen column, which leads to the tangential stresses. If the tangential

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stress is bigger than the surface tension, the cryogen column will be broken into liquid droplets or big droplets further break into small ones. But LC_3H_8 and LN_2 are both pure substances whose boiling temperatures only relate to the pressure. So the surface tension gradient along two fluids contact interface is raised from the initial degree of subcooling. In run 6, the LN_2 is nearly saturation liquid, so there should be less Marangoni effect in this process. In run 5, the LC_3H_8 has ~120 degrees of subcooling, so there should be an obvious Marangoni effect. That can be seen from the *Fig. 5*. Comparing 1a~1f and 2a~2f, the pattern of LNG bubbles have a less value of L_c/D_c than LN_2 bubbles (L_c is the length of the bubbles pattern; D_c is the diameter of the bubbles pattern). It means LNG column suffers much more tangential stress than the LN_2, which makes more bubbles break up from side than the leading end. And from 3a~3f, the LC_3H_8 bubbles generate more from side than the leading end of liquid column. It means the Kelvin-Helmholtz instability and Marangoni convection occupies leading position on the process of column breaking into droplets.

(2) The degrees of subcooling of LC_3H_8 is much more than LNG and LN_2, as a result of that, it needs more time and heat to be saturated, and in turn, the maximum pressure occurring time of run 5 (LC_3H_8) is obviously later than the other runs of 1 (LNG) and 6 (LN_2). Furthermore, the maximum pressure occurring time of run 1 is a little later than run 6. On one side, the density of LNG is less than LN_2, which causes the relative velocity of LNG is slower than LN_2. On the effect of instabilities of Kelvin-Helmholtz and critical Weber number, the intensity of breaking of LNG is not as strong as LN_2. So the maximum pressure occurring time of LNG is not as early as LN_2. On the other side, the LNG is subcooling cryogen and the LN_2 is saturate substance. In the process of LNG injection, it needs some time to heat it to be saturated, though the time will be substantially short as the difference of temperatures of LNG and water being huge.

(3) From the above analysis, the Marangoni convection has an obvious effect on strengthen heat transferring rate. Base on the instabilities of Rayleigh-Taylor, Kelvin-Helmholtz and critical Weber number theory, the maximum pressure detected in LN_2 test (run 6) should be higher than LNG test (run 1). But the real results are just the opposite. The mainly reason is the effect of Marangoni effect. As the relative velocity becoming slower and slower, the Marangoni convection takes the leading position and the instability effects of Rayleigh-Taylor, Kelvin-Helmholtz and critical Weber number theory becomes weaker and weaker. Because of the effect of Marangoni convection prolonging the breaking time of droplets, the maximum pressure and the occurring time of maximum pressure in run 1 is separately higher and later than run 6.

Bubble behaviors analysis

From these images of 1a~1f and 2a~2f in *Fig. 5*, the structure and pattern of bubbles have a similar developing process in run 1 and run 6. It can be characterized in three main stages (Zhang et al., 2015; Clarke et al., 2010; Dahlsveen et al., 2001).

(1) Stable film boiling stage: from the images of 1a~1b and 2a~2b, it can be seen that liquid cryogen flashes as it impinges into water and forms a vapor void. Because the injection process is likely to continue for some time, the following liquid cryogen is injected into the void created by the former boiling cryogen. A velocity difference forms on the relatively stable vapor film between liquid cryogen and water, but the water outside vapor film is nearly static. So the liquid cryogen inside vapor film is

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moving downwards under a high speed. There is no significant breaking on the liquid cryogen column, although formation of waves on the vapor film due to the instability of dynamics is observed.

(2) Bubbles cloud generation stage: as the liquid cryogen continuously moving downwards, on the effect of instabilities of Rayleigh-Taylor, Kelvin-Helmholtz and critical Weber number, the shearing stress is bigger than the surface tension, and then the ligaments and fragmentations take place. In the images of 1c~1d and 2c~2d, it shows the vapor film becomes thinner and lighter. There is a cloud of bubbles form on the head and the side of the column (Wen et al., 2006). The cloud of bubbles is extremely unstable; some of them will be coalescence and others will be continuously breaking up into smaller ones. The heat transferring rate will be dramatically increasing as a result of the bubbles growing up and breaking which enhances the heat convection between cryogen and water. On the effect of Marangoni convection, the images of 1c~1d show the bubbles cloud has a more unstable vapor film nearby the head of column than 2c~2d.

(3) Buoyancy taking over stage: in the images of 1e~1f and 2e~2f, it shows that, in the last stage of injections, the buoyancy force will take over. The bubbles cloud gradually disappears and forms much bigger bubbles that are so stable that they cannot be broken up again in the upwards moving process. Comparing the images of 1e~1f and 2e~2f, the bubbles cloud maintain longer in run1, because of the effect of Marangoni convection.

The images of 3a~3f in *Fig. 5* shows a different structure and pattern of bubbles. It can be characterized in two main stages.

(1) Stable flow boiling stage: in all images of run 5, the LC_3H_8 column has a relatively obvious shape and the boiling is not as violent as run 1 and 5. Most of it has no significant breaking except the periphery of the vapor film near the head of column in the image of 3d. There are three main reasons.

Firstly, the water temperature is ~303K, which is less than the superheat limit temperature of LC_3H_8. As a result of that, the explosive boiling would have less opportunity to take place and it undergoes a relatively stable boiling, that is subcooled flow boiling.

Secondly, because of the ~120 degrees of subcooling, after LC_3H_8 injecting into water, it needs much more time to be heated to the saturate temperature by surrounding water. As a result of that, it can maintain an obvious liquid column shape in a relative long time. Because the water temperature is less than the Leidenfrost temperature of LC_3H_8, the vapor film of run 6 is not as stable as run 1 and 5, and the surrounding fluid will be water. The relative velocity, which is related to density difference between LC_3H_8 and surrounding fluid, is less than run 1 and 6. Basing on the instability of Kelvin-Helmholtz and Weber number, as relative velocity decreasing, the most dangerous wavelength and minimum characteristic length will be considerably increasing. It implies the LC_3H_8 will become more and more stable and suffer less from the instability of Kelvin-Helmholtz and Weber number.

Finally, LC_3H_8 is ~120 degrees of subcooling. As a result, in subcooled flow boiling, the surface tension gradient along two fluids contact interface is raised from the temperature difference in the vapor film around the liquid cryogen column, which leads to the tangential stresses on liquid cryogen side and vapor cryogen side at the interface respectively. When the stresses accrete to higher than the surface tension, the

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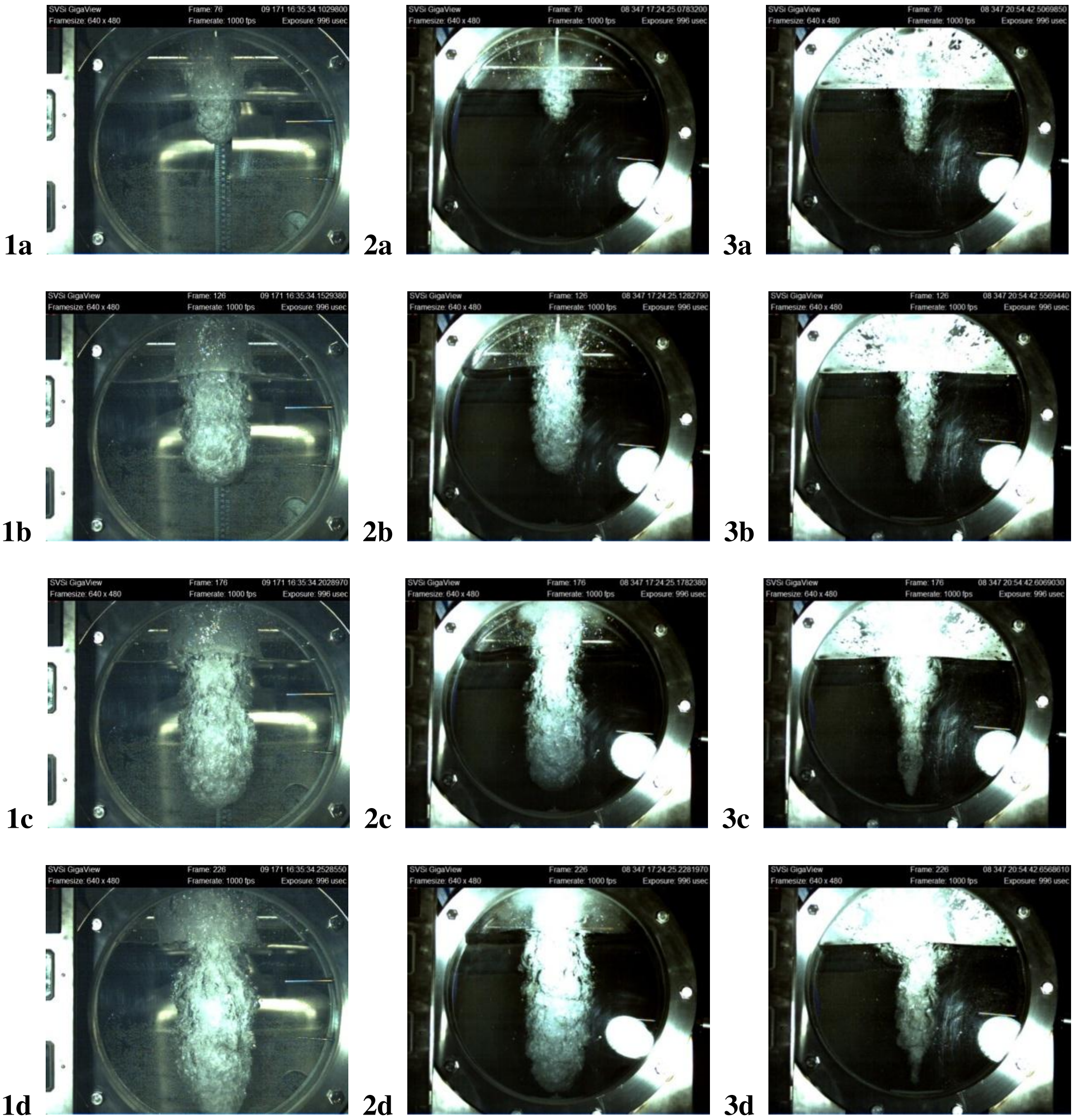
Marangoni effect will take place and some of the liquid column will breakup into small droplets, which can be seen in the image of 3d. From that image, we can also know that the periphery of the vapor film near the head of column is considerably stronger than other regions in the effect of Marangoni.

(2) Buoyancy taking over stage: in the images of 3e~3f, it shows that, in the last stage of injections, the buoyancy force will take over. The evaporating bubbles move upwards in a higher speed than the LC_3H_8 column and begin to separate from it. After that, the LC_3H_8 column still staying in the water is not broken again and some of it maintains liquid phase until floating on the water and forms liquid pool.



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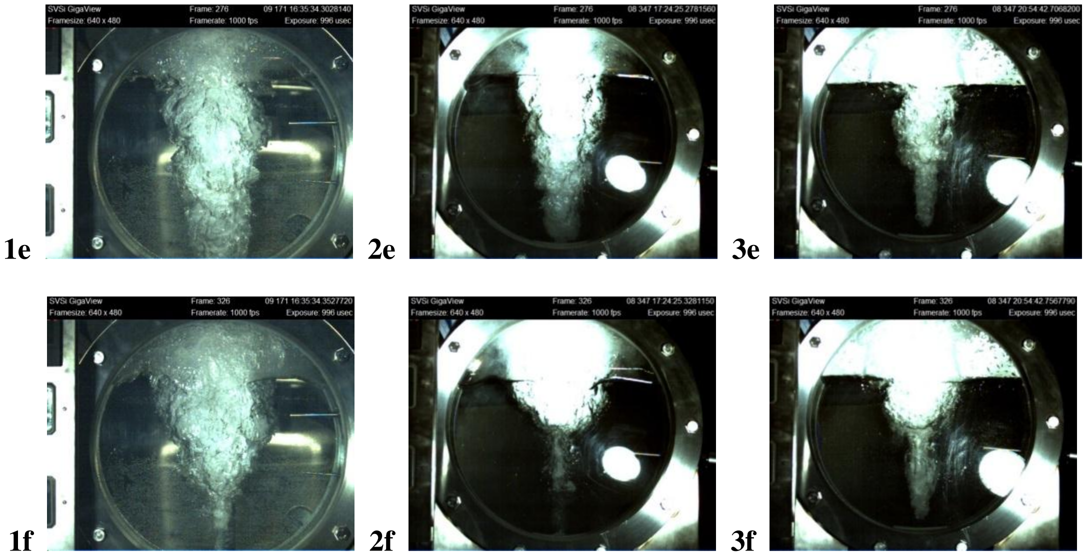

Figure 5. *Bubble behaviors of cryogen injection into water (1a~1f are images of run 1, 2a~2f are images of run 6, 3a~3f are images of run 5. The interval time between the adjacent two images is 50 ms).*

Conclusions

The process of cryogen injection into water was studied experimentally and theoretically. Main conclusions can be drawn as following:

(1) There are four main instability theories used in analyzing cryogen injection into water process. If the relative velocity between cryogen and surrounding fluid is high enough, the breaking of liquid column or droplets will be defined by the Kelvin-Helmholtz instability and Weber number theory. Furthermore, strong Marangoni effect on vapor film breaking can be obviously observed at the beginning of column floating upwards or the ending of column going downwards. And the periphery of the vapor film near the head of column is considerably stronger than other regions in the effect of Marangoni convection.

(2) When LNG is injected into unconfined water in the pressure of 7bar, the explosive boiling remarkably occurs with high heat transfer performance. The maximum heat transferring flux can be over 1.9 MW/m^2. It is ~7.8 times bigger than the theoretical limit of heat transferring flux of the pure liquefied methane nucleate boiling (0.244MW/m^2).

(3) Besides water temperature, both injection pressure and depth of injection into water influence explosive boiling occurring and its heat transfer performance.

(4) According to the images of bubble behaviors, LNG and LN_2 injection processes undergo a similar boiling, that is explosive boiling, which is characterized by bubbles cloud that strengthens heat transferring rate. LC_3H_8 injection into water triggers subcooled flow boiling. There is no significant breaking on the liquid cryogen column and without bubbles cloud.

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COUPLING MECHANISM OF REGIONAL CARBON-WATER SYMBIOSIS SYSTEM AND WATER RESOURCES REGULATION AND CONTROL UNDER LOW CARBON PERSPECTIVE

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Abstract. The purpose of this paper is to propose a new research method to solve the problem of reducing the "source" and "sink" measures in the implementation of low carbon governance, promote the development trend of "reducing sources and increasing exchange rate", and to achieve an ideal state of negative carbon emissions. This paper is based on the research of low carbon sustainable and carbon - water coupling in ecological environment management, and based on the social and economic demands, a carbon cycle system based on carbon emission and carbon capture characteristics and a water resource circulation system characterized by ecological water demand and water pollution are constructed, and the two structures are coupled to each other. The regional carbon and water symbiotic coupling system with the coupling relationship of carbon water circulation system is proposed. This paper analyzes the internal components of carbon-water coupling system and its influencing factors from the perspective of "low carbon", reveals its internal mechanism and establishes a multi-objective game decision model for water resources regulation in regional carbon-water symbiosis system. At the same time, this paper has optimized the water resources regulation and control in the pilot area. In this paper, by adjusting and perfecting the configuration of carbon and water coupling system in the regional water resources system, the "source - sink" integration in the carbon cycle is realized, which is the ideal mode of "reducing source and increasing exchange rate".
Keywords: *comprehensive evaluation model; sustainable development index; carbon-water coupling; low carbon mode; water resource rational allocation*

Introduction

Climate change has a profound impact on the supply and demand of water resources, leading to lower carbon capture capacity of ecosystems; In addition, socio-economic development leads to an increase in carbon emissions, carbon emissions increased and reduced carbon capture resulting in an integrated carbon-water system imbalance (Hannah et al., 2004; Hashem et al., 2016; Mi et al., 2014; Jiang et al., 2016; Petts et al., 2006). Although carbon emission reduction technology has been gradually promoted and applied (IPCC, 2007; Ge et al., 2015; Liu and Liu, 2010; Schramm, 2015), the ecological planning based on returning farmland to forest has also been implemented. But at present, the net carbon emissions are still in a high stage of increase, only from the past "increase sources, reduce sinks" model to "source reduction" model change, has not yet completely transition to the "source reduction and increase sinks" model. The reason is that the mitigation measures of climate change mitigation at the present stage will be separated from "reducing sources" and "increasing sinks" (China Water Resources Bulletin (1998-2007)), and lacking the comprehensive regulation and management system and methods of ecological and environmental systems of "reducing sources" and "increasing sinks". Water is a crucial environmental factor in the carbon capture and storage system of the ecological environment system. The "natural-artificial" dual water cycle changes

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the regional water conditions, affecting the formation, development and succession of ecosystems, and directly affects the carbon emissions and carbon capture processes of ecosystems, affecting the net carbon emissions of regional carbon (Feng et al., 2016; Zhang, 2016). According to *Figure 1* that the economic and social system is carbon source, the ecosystem is carbon sink. According to statistics, compared with 2000, China's carbon emissions and water consumption in 2012 respectively increased by 112.8% and 7.4% (Peng et al., 2015; National Development and Reform Commission, (2009)). Increased demand for water resources and increased demand for "fossil fuels" by socio-economic development has resulted in an increase in carbon "source" emissions and a reduction in carbon sinks. Therefore, the conceptual model of carbon-water symbiosis coupling based on carbon-water balance system is constructed, and the factors, structure and mechanism of regional carbon-water coupling system generalization model are discussed. Based on the identification of water resource, water user, carbon source and carbon sink, this paper reveals the relationship between water and water users, the relationship between carbon emission and carbon capture, and the mutual relationship between water cycle and carbon cycle, and to establish a theoretical and applied strategy for carbon-water balance to regulate and control water resources system under the effect of "low carbon".

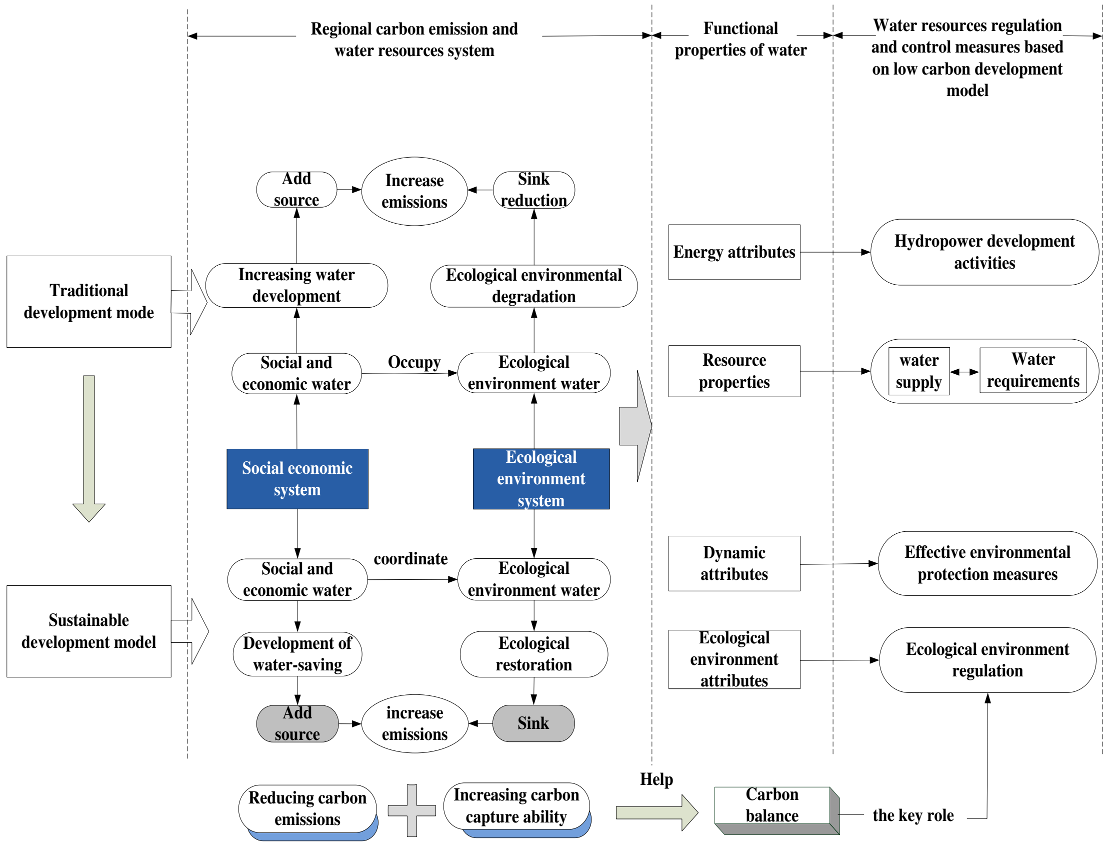

***Figure 1.** Water resources regulation and control of carbon balance mechanism diagram.*

Material and Methods

In this paper, the water quality and water quantity early warning model of regional carbon and water symbiosis mechanism is established by making use of system modeling,

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data mining, fuzzy evaluation, scenario simulation and game theory. At the same time this paper uses the system optimization decision-making modeling method, combining with multi-objective, uncertain variables of the complex programming mode, to reveal the theory and method of water resources regulation and control based on low carbon model. Specific research methods are as follows:

Analysis and conceptual model of regional water quality and water quantity correlation under low carbon

From the viewpoint of water resources system, the water circulation system under the regional carbon water symbiosis complex system should include the water quality safety subsystem and the water quantity security subsystem. Water quality security is reflected in if the urban water environment system water quality standards are compliance or not, water security is reflected in whether the water requirement sectors such as ecological environment, living and production in the case of water quality standards are able to achieve the balance between supply and demand. In addition, the regional water resources system is a complex system which takes human construction and operation management as the core. It includes several sub-systems and many components. How to describe the relationship between water quality and quantity and the interaction and evolution of the subsystems is the fundamental question. It is also a key problem to study the interaction mechanism of the water quality and water quantity system in the regional carbon water symbiosis complex system, and to construct the conceptual model of the water quality and quantity correlation system. This part of the study is divided into the following two aspects:

Correlation analysis of regional water quality and water quantity under low carbon model

Aiming at the structural characteristics of water circulation system with multi-water source, multi-user and multiple transmission and distribution water projects, the water circulation system is decomposed into multiple independent control units by the distributed control method of the complex system. And this paper analyzes the water transport and distribution relations and the pollutant migration and transformation rules of each control unit. The regional carbon water symbiosis complex system was introduced into the analysis of water quality and quantity connection effect. The simulation results are used as the main information source to analyze the regional water quality and water quantity movement mechanism, establish the regional carbon water symbiosis complex system water quality quantity water correlation relations. In practice, researchers will further improve and perfect the joint regulation of water quality and quantity of water resources.

Establishment of the conceptual model of regional water quality and water quantity correlation under low carbon

It will improve the current water quality studies coupled commonly used "loosely coupled" structure, that is quantity and quality in the system as independent of each subsystem, and then turn the water quality modeling and simulation method. In this paper, we consider the coupling between water quality and quantity, the coupling and interaction between the regional water cycle system and the economic and social water use system from the aspects of the interaction between the artificial - natural binary

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circulation, the integrity of the watershed, the target control at the macroscopic level of the watershed and the concrete simulation of the microcosmic aspect of the river basin and then on the basis of clarifying the rules of water transfer and water quality variation among different parts of the system, a conceptual model of water quality and quantity association system in regional carbon and water symbiosis complex system was established, and the water quality and water quantity correlation system in the study area was comprehensively evaluated, this is the basis of the follow-up research.

Identification of carbon - water symbiosis mechanism and theory and method of system comprehensive evaluation under low - carbon

At present, the theory and method of rational allocation of water resources based on low carbon development model are still in the exploratory stage, and there is no mature model available at home and abroad for reference. This paper summarizes the domestic and international water resources allocation theory and technology research new progress on the basis of the proposed low carbon development model for the rational allocation of water resources mechanism of carbon water symbiosis system to multi-objective decision-making and simulation technology as the key support, water resource regulation and control model of carbon - water symbiosis system from low carbon perspective. The specific research methods adopted are as follows:

Identification technology of operation mechanism of regional carbon-water symbiosis system under low carbon mode

The prerequisite and basis of regional carbon-water symbiosis system is identification of system configuration, in which the identifying objects include the water circulating system under universal concept and the carbon cycling system based on carbon source and carbon sink (Socio-economic system mainly for the carbon emissions of the system, while the ecosystem is mainly manifested as carbon-capture systems). The core of this part of the research is to draw the model of network generalization of Regional Water Resources System based on the carbon-water symbiosis of coupled system based on the distribution of the existing carbon-water circulating system in the experimental area, based on the system identification of water source, water user, carbon source and carbon sink. The model of network generalization of Regional Water Resources System (shown in *Figure 2*), through the system network model method to establish the configuration of unit and the node, the system to establish the relationship between water and water users, the relationship between carbon-emissions and carbon-capture and the constraint relationship between water cycle and carbon cycle.

The theory and method of comprehensive evaluation of regional carbon and water symbiosis system in low carbon mode

It is necessary to establish a reasonable and effective evaluation system based on the above-mentioned regional carbon-water symbiosis system, the main task of the research is to study the control planning method and management strategies of water resources under the low carbon perspective with the system optimization and decision analysis method, the main research tasks include: Selecting the evaluation index of the research object, establishing the evaluation mathematical model, determining the key parameters of the model, solving the model and analyzing the result. The selection of evaluation index is the emphases and key point.

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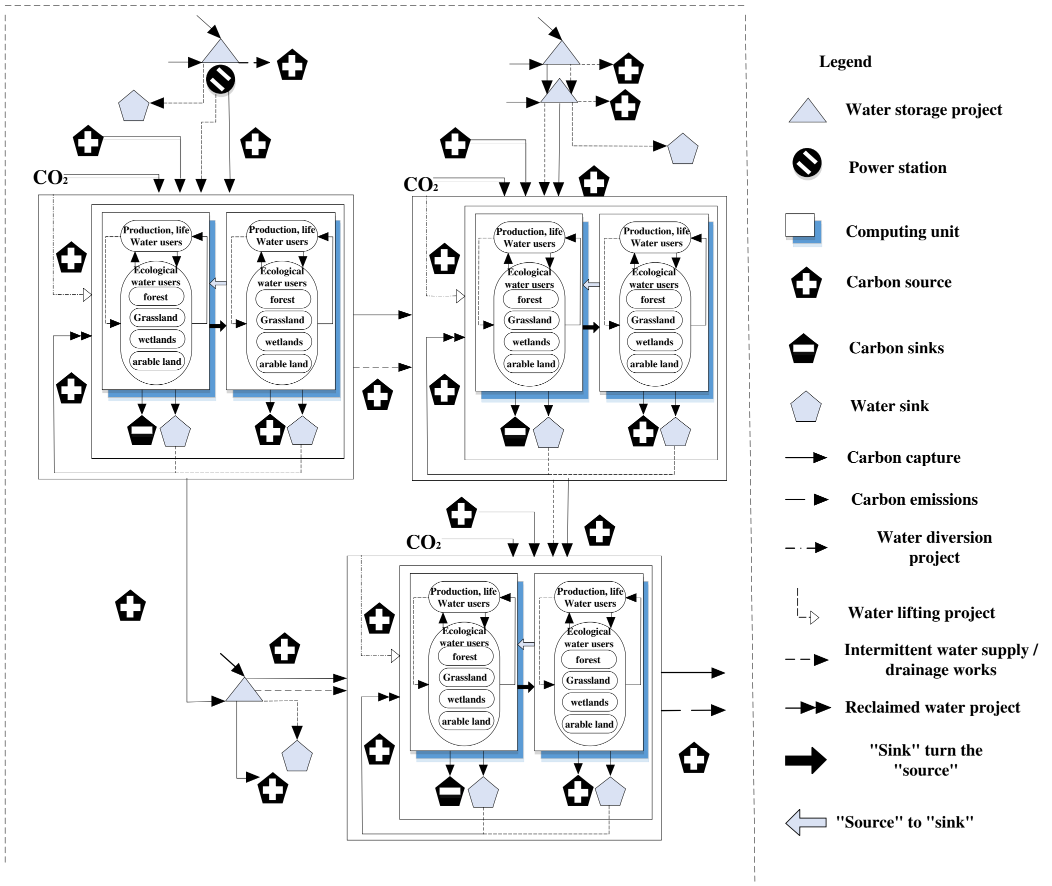

Figure 2. *Regional carbohydrate generalized model symbiotic system schematic.*

Water quality and water quantity simulation of regional carbon - water symbiosis mechanism and safety early warning technology of water circulation system

Through the analysis of the literature, it is found that the previous studies on the simulation of water pollution are mostly carried out under a single static characteristic, which is not consistent with the objective reality. Because the practical meaning of the water cycle system with "multiple fuzzy" complex features: Uncertainty (Spatial and temporal distribution unevenness of water), Dynamic (The dynamic of regional ground confluence changed follow the time), Irregularity (Irregular nature of socio - economic water use). Therefore, it is reasonable and practicable to study the regional water quality simulation and early warning system in combination with the occurrence, development and spread of dynamic evolution disaster under the "multiple fuzzy" characteristics mentioned above.

Construction of water quality and quantity simulation model based on scenario simulation of regional carbon and water symbiosis mechanism

First of all, it is to collect extensively with all types of hydrological data which based on the test area, and regional geographical environment, the main water supply facilities, drainage network and channels, precipitation and other spatial and temporal distribution of data. And at the same time to identify the influencing factors and processes of water quality and water safety under the regional carbon and water symbiosis mechanism;

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Based on the historical and literature data, the multiple fuzzy features of the main driving factors of the system are analyzed; On this basis, the uncertainty assumption method is used to set up hypothetical condition of the evolution of the environment, which can be used as the input of water modeling and water pollution simulation model construction of regional carbon-water symbiosis mechanism, and systematically identify water sources, pollution sources, carbon "sources" and carbon sinks, and establish allocation units and nodes to establish a database of water sources and pollution sources, carbon emissions and carbon capture, and water cycle and carbon cycle system interaction and status information, and combined with the Petri Net rule model described as the scene data source generation tool, as the establishment of water quality and water quality of the set of elements of the background information.

Pre-warning technology of water quality and water security system based on scenario prediction model of regional carbon-water symbiosis mechanism

Finding the historical water quality and quantity database of the experimental area and using the multi-dimensional correlation function to solve the problem of nonlinear mapping between the water security risk factors and the key early warning indicators, and obtain the connection function of the water quality and water quality between risk factors and warning indicators through the fitting calculation; Based on the historical data, the safety factor and the early warning classification correlation model were established to determine the water pollution classification type, the early warning control parameter and the key early warning index threshold ; The establishment of a diagnostic model will change with the diffusion time of the key indicators of police intelligence and early warning indicators to assess the threshold value of tracking and comparison, once the alarm threshold is exceeded signal (*Figure 3*), and input to the corresponding early warning level deduction model to realize the logical structure of early warning response, and provide the guarantee for regional water resources regulation and control.

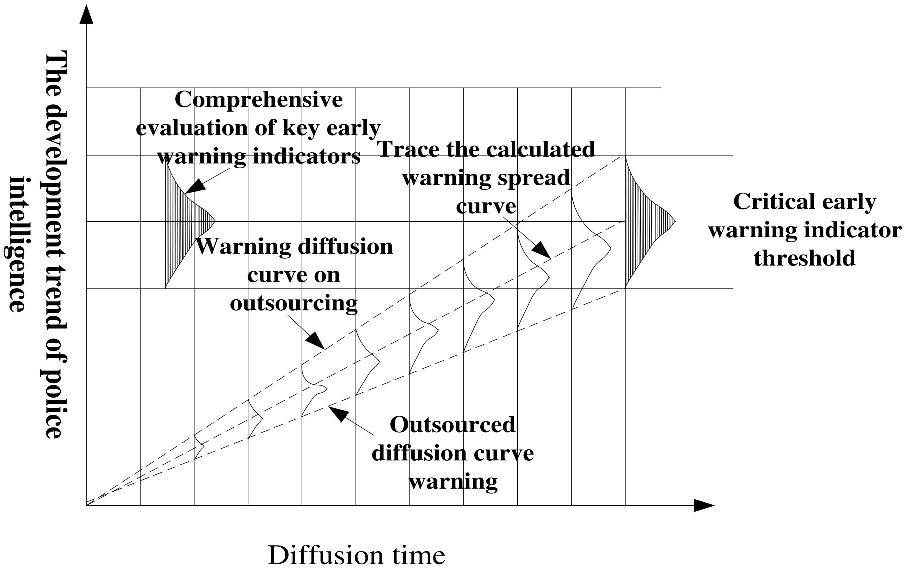

***Figure 3.** Key early warning indicators threshold curve based on tracking comparison.*

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Regulation and Control of Water Quality and Quantity in Regional Carbon and Water Symbiosis System from Low Carbon Perspective

Regional carbon - water symbiosis mechanism identification and comprehensive evaluation of the system and safety pre-warning of the water cycle system provide a basis for the regulation and control of water resources based on the coupling mechanism of regional water quality and water quantity under low carbon mode. However, how to plan and manage regional carbon-water symbiosis system to optimize the water supply regulation and water pollution control of regional water management departments to determine whether the carbon-water circulation coupling system of the study area is balanced, and to determine compliance with regional water bearing capacity safety standards is a regional carbon water symbiosis system water quality and water regulation and control of another important prerequisite. This article mainly studies according to the following two aspects.

A multi-objective game decision-making model of water quality and water quantity regulation in regional carbon water symbiosis system under low carbon perspective

Considering the economic, environmental and social factors of the test area, this paper puts forward the idea and method of constructing a multi-objective game decision-making model of water quality and water quantity regulation in regional carbon water symbiosis system under low carbon perspective, and designs the model structure based on multi-objective game analysis and the model solving method of co-evolutionary algorithms, and selects the water resources system of the typical city in the test area to model. Based on the theory knowledge and technology of system, optimization decision, modeling method comprehensively, this paper adopts the method of multi-objective decision-making and simulation and uses decision-making and simulation methods to set middle and long-term water resources regulation sequence elements, and gives water resources allocation under different scenarios of supply and demand, water quality control and carbon emission reduction.

Formulating regulation and control measures of water quality and water quantity regulation in regional carbon water symbiosis system under low carbon perspective

Based on regulation and control methods of water resources in regional carbon water symbiosis system under low carbon perspective is a multi-source, multi-carbon source and multi-control factors integrated water resources management model. In this paper, the water resources regulation model based on low carbon development model is used to analyze the schemes, optimize the comparison and output the regulation scheme. Then, according to the key assumptions of the carbon-water symbiosis system, the results are used to construct the system evaluation and evaluation system, and the recommended scheme is analyzed and evaluated. Then the article gets the recommended governance solution. Finally, based on regional general situation, this paper puts forward practical and feasible measures to ensure water quality and water quality of regional carbon-water symbiosis system under low carbon perspective.

Results and Discussion

First of all, this paper analyzes the correlation and transformation between the various components stem from the components of the regional water quality and water quantity system and its influencing factors. In order to establish the conceptual model of regional water quality and water quantity system and reveals its internal influencing mechanism, this provides the base of qualitative and quantitative research for carbon water symbiosis system. Secondly, on the basis of the characteristics of carbon-water coupling system, under the principle of "low carbon" and "sustainable", considering various factors that "Human - Carbon - Water" Realistic Environment has an impact on the safety and sustainability of regional water resources systems. The effect of multi - factors on carbon - water symbiosis system was discussed, and the mechanism of regional ecological, environmental, economic and social systems under low carbon mode was revealed. Furthermore, the constituent elements of the regional water resources system and their influencing factors were proposed according to the carbon-water symbiosis system model. The interaction between the components was analyzed and the water quality prediction model based on water pollution simulation and evaluation was established. , To improve the water supply security, water security. Finally, through the modeling and case analysis of the regulation of water resources in regional carbon - water symbiosis system, this paper reveals the mechanism of Regional Carbon-Water Coupling System and the improvement of water resources regulation and control measures under the View of "low carbon", and providing a scientific and reliable technical support for the river basin water system governance.

Conclusion

In this paper, the Haihe basin is considered as a research area, taking the conceptual model and the role identification of carbon-water coupling system into account. Through systematic evaluation and simulation, the characteristics of carbon and water cycle and water resources system are identified, and the regional carbon emission benefit, carbon capture efficiency and carbon emission net benefit divided by basin were focused. Design and establishment of water security early warning model under the low carbon effect model, in order to meet the requirements of water resources management and climate change comprehensive response under the conditions of low carbon and water security, this paper, based on the water security coordination control method in the field of environmental governance, scientifically allocates the water resources of the river basin. Finally, this paper presents a new idea and new method to solve the water resources regulation and control in the carbon-water symbiosis system.

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A PATH ANALYSIS OF THE IMPACTS OF VALUE TRANSFER ABOUT FOREST ECOLOGICAL SERVICE FROM UPPER WATERSHED OF HUNHE ON WATER SUPPLY IN SHENYANG CITY IN CHINA

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Abstract. The ecological service (ES) of soil and water conservation that is provided by the forest ecosystems in the upper basin of Hunhe river has some movement in certain space, which has an important influence on water supply in Shenyang city in China. From the perspective of forest ES transfer, it works out the ecological service value of transferring in the upstream of Hunhe river towards Shenyang city from 2010 to 2014 with the help of Buffer and Intersect of ArcGIS9.3, and the path analysis is used to explore the impact of the ecological service value (ESV) of transferring in the upstream of Hunhe River Basin on the water supply in Shenyang city. The results demonstrate that firstly total values of transferring from 2010 to 2014 adds up to 5.647 billion RMB, and secondly the ecological service of soil and water conservation has positive external effects on the water supply in Shenyang city by the surface water, groundwater and chemical oxygen demand. It can be seen that it explains the mechanism of action about the impact of transfer value of forest ES in the upper watershed on the water supply in the lower basin, which provides the theoretical foundation to the quantitative analysis of value transferring of forest ES and has an important proof of payment for ecological services of river basin in the theory.
Keywords: *path analysis; ecological service (ES); transfer value; water supply; Hunhe River Basin*

Introduction

It is well known that human well-being depends on a variety of ecosystem services (ES) provided by nature, ranging from supporting services and provisioning services to regulating services and cultural services. Ecosystems face various pressures resulting from urbanization and increasing population. Especially, the availability and quality of water for many regions in China have become more and more threatened by shortage and pollution. China's per capita water is less than 25% of the world's per capita level. According to the statistics by the Ministry of Water Resources, there are more than 400 cities in 655 cities in China, which have water shortage of 0.16 billion m3/d, especially severe water shortage in Beijing and Tianjin. As a result of frequent drought, extreme climate change, and more than 50% of rivers and lakes contaminated, the lack of water supply becomes more and more serious (Liao et al., 2016). And fortunately, it has been increasingly recognized that shortage and pollution of water are strongly influenced by forests. The forest is playing an important role in regulating water quantity and purifying water quality. Ecologists argue that riparian forest is widely regarded as temporary reservoir capturing and processing energy to provide water resources for human beings and maintain various ecological functions. In order to maintain ecological service function in river basin, payment for watershed ecosystem services (PWES) has

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been used to encourage riparian residents from upstream to protect water resources in many countries. For example, the hydropower company funds afforestation in upstream in Costa Rica, and Irrigation Association of Kauka River Basin in Colombia pays fee for forest from upstream to regulate river runoff. Those measures for tree planting and conservation of vegetation are implemented by the payment for watershed ecosystem service to regulate and purify water resource (Zhen, 2006). Therefore, the relationship between forest service value transfer and water is a critical issue.

The services of ecological systems and the natural capital stocks are very critical for the planet's life-support system. They contribute to human welfare, both directly and indirectly, and therefore represent part of the total economic value of the planet (Costanza, 1997). Ecosystem services have being an important concept widely used by ecologists since the 1990s, some scholars have divergent views on the concept of ecosystems services. Ecosystem services are the conditions and processes that natural ecosystems and their constituent species can be used to sustain and meet human survival (Daily, 1997). Costanza defines ecosystem services are that the benefits that humans derive from ecosystems (1997). Ecosystem services refer to the ability of ecosystems to provide a variety of products and services to humans in the Millennium Ecosystem Assessment (MA) (2003). To a certain degree, Wallace agrees with the definition identified by MA, but there are some differences. He stresses that ecosystem process is not the ecosystem service but the production mode of ecosystem services (2007). The definition of ecological services is different among many scholars, it should be defined according to the specific purpose (Zhang, 2012). The ecological service system should be regarded as an entirety, its ecological service functions are interrelated and interacted each other under certain conditions. The concept of ecological service proposed by MA would be adopted in this paper, it classifies the ecosystem serviceswhich function of water purification and water regulation provides the theoretical basis to analyze the impact of watershed forest on water resources.

For a long time, ecosystem services are seen as inexhaustible and free public services, it causes the scarcity of ecological service supply and excessive consumption of ecological services (Egoh et al., 2007). Ecological assets per capita are scare in China, it is an urgent for the natural resource management and ecological compensation to fully evaluate the value of ecological services (ESV) (Xie et al., 2015). The evaluation of ESV is broadly regarded as a useful tool to monetize and measure ecological services. In recent years, many scholars have used static evaluation methods to assess ESV. The contribution of global ecosystems to human welfare is assessed by the static equilibrium model (Dou, 2003). This model usually uses shadow prices and shadow values to estimate the total ESV, but it is based on the assumption that the value of ecosystem services is limited by its direct and indirect impact on market value so that it can be underestimated (Xie, 2011). There are some shortcomings for the accuracy of the simulation results in the biodiversity model due to simplified assumptions and lack of uniform evaluation indicators (Li et al., 2013). Traditionally, cost-benefit analysis, firmly grounded in economic theory, is always a major tool to assess ESV, although it is restricted to the need for the monetised inputs. Some economists believe that the core of valuation for resource, environment and ES is the consumers’ preferences. At present, the Contingent Valuation Method (CVM) is also a major tool to assess the non-use value of environmental goods and ES (Carson, 2000) by the respondents’ willingness to pay (WTP) or willingness to accept (WTA). In 1947, Ciriacy-Wantrup proposed by direct inquiry to understand the respondents 'willingness to pay for public goods and to

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compensate the ecological environment, and then estimate the economic values of public goods and ES (Portney, 1994). Xu takes Liaohe River Basin as a research object and calculates WTP and WTA of the respondents among the residents and analyzes the disparity by using CVM (2015). The close-ended questionnaire and open-ended questionnaire are basic survey techniques for the CVM, but the evaluation result of the two techniques have been controversial, which main argument is that the former obtained WTP higher than the latter. Hanemann argues that the reason of the difference between the two technologies is that respondents' cognition for ES and public environmental goods (1994). So the ESV estimated by CVM are characteristic of subjectivity and greater deviation. As these models mentioned above statically or subjectively evaluate the ESV, they are not suitable to assess the ESV in river basin, considering that the values of some forest ES is flowing and transferring through a certain medium in the space (Qiao et al., 2011).

There are two main methods for evaluation of forest ES, namely, the value evaluation method and material evaluation method (Hu, 2007). Xiao evaluates qualitatively their economic values of forest ES from 2006 to 2011 by the market value method and production cost method (2014). The disadvantages of these two methods are difficult prediction about the level of ecosystem supply, misunderstanding of the link between the evaluation object and the marketable commodity and difficulty of cost statistics. While the assessment of forest ESV by material evaluation method is based on the existing unit area of ESV multiplied by the corresponding area, and the unit area value of forest ESV is calculated by the Net primary productivity (NPP). Ecosystem services are directly related to the biomass. The productivity and service value of ecosystems is estimated by a series of simplified models based on large-scale remote sensing information. It provides the method for measure of ESV, which has higher accuracy (Paula, 2012). It is common to establish the relationship between forest parameters and the Geographic information system (GIS) (Huang, 2015). Although there are several popular application software that are available in the market such as GRASS GIS, SAGA, gvSIG, ArcGIS have been widely adopted in many fields such as business, scientific research and resource management. ArcGIS is the software of spatial information analysis, it could be not only used to collect data, store data, analyze data and monitor the spatial changes of forest areas, but also measure transferring value of interregional ES, and analyze the impact of different ES. Environmental value transfer is commonly defined as the transposition of monetary environmental values estimated at one site through market-based or non-market-based economic valuation techniques to another site (Brouwer, 2000). Environmental value transfer as the procedure estimated on the unobserved monetary value has been widely used, ranging from water quality management to forest management (Luken, 1992; Bateman, 1995). Though the value transfer technique is widely applied in developed countries, it is seldom used in the developing countries, due to the lack of ES valuation expertise (Chaikumbung et al., 2016). The forms and driving factors of value transfer analyzed are necessary supplement to the studies of ES evaluation in China (Fan et al., 2007). Take Weigan river basin in China as an example, it is a significant attempt to do the value transfer estimation of SE by building transfer assessment model of ESV and suing ArcGIS9.3 buffer and overlay analysis platform. The results indicate that ESV has increased continuously from the upstream to the downstream during twenty-two years (Qiao et al., 2011). Besides, value transfer also contributes to determining ecological compensation standard and forming different levels of these in a particular situation (Chen et al., 2014).

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The forest is the key to clean water. A high proportion of the Nation's freshwater resources originate from forests. The relation between forest and water also remains controversial. In the past, there was the assumption that forest not only can maximize water yield, but also regulate seasonal flows and improve water quality. Although the forest is not to significantly increase downstream water yield, the critical role of forest covering upper watershed for ensuring water supply and the delivery of high-quality water has been confirmed (Bruijnzeel, 2004; Calder, 2007; Van et al., 2007). The role of forest in regulating stream flow and groundwater are very effective through interception and evaporation of precipitation from the tree canopies and transpiration from the foliage, and then transportation by litter layer and soil layer (Zhou et al., 2002). In addition to these ecological services, purifying water is also significant by trapping or filtering other water pollutants to alleviate the sediment in water bodies. For instance, the broad-leaf forest ecosystems in the upstream of Jinshui river basin in China make their most significant contribution to regulate the PH value and alleviate various physical-chemical concentrations so as to cleanse water (Bu et al., 2010). The forests in the upstream is maintaining water flows during the dry season and cleansing water supply, which could provide water-quality resources for residents in downstream. The existing literatures only analyze the direct impacts of forest in the upstream of the basin on water resources, and its indirect effects cannot be further analyzed. Path analysis provides the effective method to study the direct and indirect impacts of forest in the upstream on water resource of the downstream. In 1972, the path analysis method was proposed by Wright. Path analysis is commonly regarded as a multivariate statistical technique, which is used to analyze the relations between two variables, and describe the impact of independent variable on dependent variable. Compared with the multiple regression model, path analysis can explain the direct and indirect effects of independent variable on dependent variable (Li et al., 2011). In addition, value transfer of forest resources in the upstream is not marketed and thus their value to water is often ignored in development decision. The complicated relationships are always a hot issue between forest and water in the river basin (Li et al., 2001), but water supply in the downstream influenced by value transfer of forest SE from the upper watershed is seldom studied. So the importance of ES value transfer in the upstream to water in the downstream will be discussed in the paper. Path analysis is adopted to analyze and reflects the direct effect and indirect effect about forest of ES Value Transfer in the upstream of Hunhe on Water Supply in Shenyang City.

Based on the facts mentioned above, from the perspective of forest ES transfer, forest ES value transfer from upstream is calculated with ArcGIS9.3 buffer and overlay analysis platform from 2010 to 2014, and then the effects of forest ES value transfer on water supply in Shenyang city cluster can be studied using the path analysis. The objectives of this research are: (i) to measure forest transfer value in both upstream and Shenyang city, and (ii) to analyze the influence of forest value transferred on water supply in Shenyang city by the path analysis. To accomplish the first objective the study performed the following tasks: (1) calculation of forest ESV in the upstream and in Shenyang city respectively, (2) determination of transfer radius and transfer scope, (3) construction of forest ESV transfer assessment model.

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Definition of forest ES transfer types in river basin

Forest ecosystem has a variety of ES functions, covering gas regulation, climate regulation, water regulation, soil formation, water conservation, water purification and so on. But it plays an important part of water regulation, water purification and water and soil conservation for forest ES in the river basin. It is highly definite that transfer scopes, influencing factors and features are various among different ecosystem services, on the basis of the definition of transfer types about forest ecosystem service in river basin (Groot, 2002) (*Table 1*). A variety of ES may be transferring in space, covering climate regulation, water conservation, soil formation and disposal of waste in the forest ecosystem. It is in river basin that forests have been recognized as a ES carrier, which water regulation, water purification, and soil and water conservation could not only be transferred in space, but also their ecological values are gradually decreased with increasing distance of space.

Study area

Hunhe River Basin lies between Fushun and Dashiqiao and crosses four cities: Shenyang, Anshan, Dengta, Haicheng, covering around 11480 km^2 (*Figure 1*). The upstream and Shenyang city in Hunhe River Basin is demarcated by Da Huofang reservoir. The upstream refers to three counties, including QingRMB, Xinbin, Fushun, while Shenyang city covers five districts: Sujiatun, Tiexi, Shenhe, Heping and Hunnan, covering 5.06 million of people. Shenyang has the semi-humid temperate continental climate, its average temperature of 8.3, its annual rainfall reaches 500 mm, its annual frost-free period is about 183 days, its forest cover rate is only 28%.

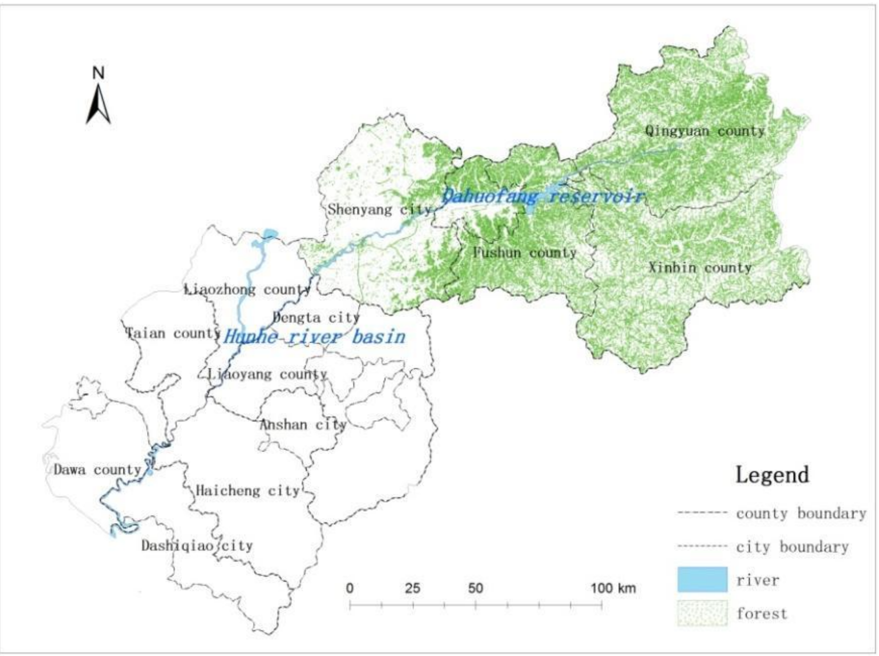

Figure 1. *Forest resources distribution between upstream and Shenyang city in Hunhe River Basin*

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Table 1. *Transfer scopes, influencing factors and transfer features of different ecosystem services*

Ecosystem service	Transfer scope / km	Influencing factors	Transfer characteristics
Gas regulation	$10^2 10^4$	The regulation of O_2、CO_2 and SO_2	With air as medium, attenuation in the circulation
Climate regulation	$10 10^3$	Albedo coefficient, heat capacity and other properties	With air as medium, planar attenuation in the circulation
Water regulation	$10 10^2$	The mesoclimate and other factors	With air as medium, planar and linear attenuation gradually
Soil formation	—	Plants, animals, microorganisms and the forces of nature	The flow and attenuation does not occur generally
Water conservation	$10 10^2$	Forest vegetation, soil , etc	According to the linear flow
Soil and water conservation	10^2	Plant roots and the forest vegetation	With soil vegetation as medium, planar attenuation gradually
Water purification	$10 10^2$	The biological and physical chemistry of water and vegetation	With water, forest and soil as medium, planar attenuation gradually
Water content regulationdisaster prevention	$10 10^2$	The degree of vegetation structure to flood and rainstorm	Planar and linear attenuation gradually
Nutrient cycling	10^3 above	C, N, S, P and other nutrients to life	Irregular, but no attenuation generally
Pollination	$10^{-2} 10^2$	The atmosphere, water, or insects	Both inside and outside the domain of irregular flow
Biological control	10^3	Various factors within the system	Without obvious attenuation in the circulation

Shenyang city has a serious shortage of water resources. Its water resource per capita is approximately one-fifth of nation's average water supply. To address this challenge, there is a need to understand the interactions between forests and water. The relationship between forest and water is based on the assumption that under any hydrological and ecological circumstance. Forest in upstream has been confirmed that help regulate seasonal flows, maintain water yield and cleanse water quality. Following this assumption, increasing forest cover in upstream of river basin is the best effective measure to ensure high-quality water for agricultural and industrial uses, as well as for residents' drinking-water supply in the downstream areas. As foresters are increasingly

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committed to conservation and construction of forest in the upstream watershed to implement sustainable forest management, it is nearly 2.82 thousand hectare of forest in 2005, and by 2015, an additional 4.13 thousand hectare of forest increased, forest cover reaching more than 66%, due to the conservation and construction of forest in the upstream watershed. In addition, there are various types of forests planted in the upstream watershed to maintain a variety of ecological functions. High-quality water obtained by Shenyang city is attributed to a high proportion of the forest cover, various types of forest, and varying ES functions in the upstream.

Data sources

Forest types from 2010 to 2014were obtained from Fushun and Shenyang Forestry Bureau of Liaoning Province in China. Forest areas in Hunhe River Basin are calculated by studying the land-use map of Liaoning province from 2010 to 2014, the related figures of forest cover comes from Fushun Statistical Yearbooks. The data of water resource data covering stream flow and ground water, chemical oxygen demand (COD) andwater supply come primarily from Shenyang water Resources Bulletin (Shenyang Water Conservancy Bureau, 2010-2014). The standard-reaching rate of water quality is from Liaoning Statistical Yearbooks (Forestry Department of Liaoning Province, 2010-2014).

Assessment of value transfer about forest ES in Hunhe River Basin

Assessment of forest ecological service value

There are many ES transferring in space, covering climate regulation, water conservation, soil formation and disposal of waste in the forest ecosystem. Based on the identification that under particular hydrological and ecological circumstance, forest ES in Hunhe River Basin can be defined as the water regulation, water purification, and soil and water conservation in terms of the transfer features and influencing factors in this paper. The forest types in the upstream of the Hunhe River Basin and Shenyang City covers the Querccus, Larix gmelinii, Pinus Koraiensis, Pinus Tabuliformis, Tilia tuan, Pterocarya stenoptera and broad - leaved mixed forest (*Table 2*).

Table 2. *Current situation of forest resources in Hunhe River Basin (Unit:10^4 ha)*

District	The Quercus	Larix gmelinii	Pinus koraiensis	Pinus Tabuliformis	Tilia tuan	Pterocarya stenoptera	Broad-leaved mixed	In total
Qingyuan County	12.88	10.87	0.58	0.43	0.16	—	2.95	27.87
Xinbin County	15.59	7.84	0.62	0.58	0.21	—	5.36	30.20
Fushun County	5.30	3.32	0.27	0.70	0.07	—	1.78	11.44
Subtotal	33.77	22.03	1.47	1.71	0.44	—	10.09	69.51
Shenyang City	—	1.38	1.34	1.22	—	2.96	0.50	7.40

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Studies have shown that biomass and net primary productivity (NPP) is a key point to assess ES value (Li et al., 2015). Total biomass of different types is calculated in the present study based on the formulation given by Fang (*Table 3*) (1996), and the net primary productivity (NPP) of forests and ES value could be calculated respectively for different forest types (*Table 4*).

Table 3. *Forest ES value provided by different forest types in river basin (Unit: 10^4 RMB, ha)*

Forest types	Water regulation value	Water purification value	Soil and water conservation	Total value per hectare
The Quercus	0.76	0.57	0.26	1.59
Larix gmelinii	0.87	0.58	0.26	1.71
Pinus koraiensis	0.87	0.58	0.26	1.71
Pinus tabuliformis	0.87	0.55	0.26	1.68
Tilia tuan	0.76	0.51	0.28	1.55
Pterocarya stenoptera	0.75	0.43	0.28	1.46
Broad-leaved mixed forest	0.76	0.63	0.27	1.66
In total	5.64	3.85	1.87	11.36
Average	0.81	0.55	0.27	1.62

Notes: ES value of the Quercus, Pterocarya stenoptera and Broad-leaved mixed forest based on the studies given by Baiand Larix gmelinii, Pinus koraiensis, Pinus tabuliformis and Tilia tuan based on the studies given by Ouyang (2011).

Table 4. *Relationship between blomas (x) and net production (y) for different forest types*

Forest types	Function expression	Biomass (x)	Net primary productivity (NPP, y)	Functional adjustment coefficient (K_i)
The Quercus	y=8.85	—	8.85	0.83
Larix gmelinii	y= -0.018x+14.294	98.74	12.52	1.18
Pinus koraiensis	y= -0.018x+14.294	76.15	12.92	1.21
Pinus tabuliformis	1/y=5.71/x+0.047	102.78	9.71	0.91
Tilia tuan	y=8.85	—	8.85	0.83

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Pterocarya stenoptera	1/y=12.092/x+0.048	268.71	10.75	1.01
Broad-leaved mixed forest	y=0.208x+1.836	49.54	10.22	0.98

NPP is a good indicator for ecosystem services and biomass, although it is limited by the need. In response to this need for the information on the value of ecosystem services about the impacts, there is a correction factor being applied into this paper (*Table 4*). K_i is computed as:

$$K_i = NPP_i / NPP_{mean} \quad \text{(Eq. 1)}$$

Where K_i is the correction factor; NPP_i represents the net primary productivity of forest type *I*; NPP_{mean} is average net primary productivity of all forest types.

According to the correction factor of forest ecological service, total values of forest ecosystem are calculated (*Table 5*) as follows:

$$V = \sum_{i=1}^{n}\sum_{j=1}^{m} S_j E_{ij} K_i \quad \text{(Eq. 2)}$$

Where V represents the ES total value, *i* is ES type;*j* is the forest type; S_j is the forest area of type *j*; E_{ij} is the ESV of *i* type provided by forest type *j*; parameter K_i is the correction factor.

Table 5. *ESV both in the upstream and Shenyang city (Unit: 10^9RMB, ha)*

District	**Water regulation values**	**Water purification values**	**Soil and water conservation values**	**Total values**
Qingyuan County	15.97	22.54	7.29	45.80
Xinbing County	16.79	23.10	7.62	47.51
Fushun County	6.42	8.95	2.93	18.30
Subtotal	39.18	54.59	17.84	111.61
Shenyang City	4.07	6.41	2.10	12.58

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Assessment of transfer value for forest ES

Riparian forests play an important role in regulating water flows and filtering and trapping the sediment and pollutants for ecological environment of river basin. The effectiveness of riparian forests in maintaining downstream water yield and filtering pollutants in a headwater catchment depends on some water-related functions of forest, including water regulation, water purification and soil and water conservation. These services transferred have an impact on the availability and quality of water in Shenyang city. So it is necessary to study the interactive relation between forest and water, for example, assessing effects of transfer value on water quality and quantity.

In geographic theories, the gravity model, breaking-point model and field intensity formula are often introduced to explain the distance attenuation principle and then to calculate transfer value of ES. In 1949, Converse proposed the concept and calculation method of breaking-point (Han et al., 2010). The breaking-point model, which also has been applied in calculation of transfer radium, can't be used in directly and need to be complemented with distance measures from between core point of forest ES in upstream and breaking-point in the downstream areas (Mooi and Sarstedt, 2011). Transfer radium A_{ij} is calculated as:

$$A_{ij} = \frac{D_{ij}}{1+\sqrt{V_j / V_i}} \quad \text{(Eq. 3)}$$

Where *i*, *j* respectively represents the upstream and Shenyang city; A_{ij} is the transfer radium; V_i, V_j is respectively the forest ESV in both upstream and Shenyang city; D_{ij} is the central distance from upstream to Shenyang city.

Transfer value of forest ES is commonly defined as the transposition of monetary ESV estimated through market-based or non-market-based economic valuation techniques. It relies on several factors, including transfer scopes measured with ArcGIS9.3 buffer and overlay analysis platform, central distance between different regions and ESV of affected areas. Transfer value of forest ES can be calculated (*Table 6*) so described as:

$$V_{ij} = P_{ij} \frac{V_j}{D_{ij}^2} S \quad \text{(Eq. 4)}$$

Where V_{ij} represents the transfer value of forest ES from the upstream to Shenyang city; S is the transfer scopes of forest ES; V_j is the forest ESV in Shenyang city, D_{ij} is the central distance from upstream to Shenyang city; parameter P_{ij} is a some factor, which ensures that medium measure ranges between 0 and 1, and it is in general 0.6 for ordinal influencing factor.

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Table 6. *Transfer value of forest ES from the upstream in Hunhe River Basin from 2010 to 2014 (Unit: 10^9RMB, ha)*

Years	Water regulation	Water purification	Soil and water conservation	Total values
2010	5.51	3.50	1.80	10.81
2011	5.52	3.51	1.81	10.84
2012	5.65	3.59	1.85	11.28
2013	5.88	3.75	1.93	11.60
2014	6.08	3.87	1.99	11.94

Path analysis about effect of transfer value on water supply

Path analysis method is to analyze the relations between two variables by decomposing the correlation coefficient, and then express the direct effect of an independent variable on the dependent variable, and the indirect effect of an independent variable on the dependent variable caused by other variables (Fu, 2014).

Path coefficient is an important indicator for the path analysis to reasonably explain the complex relationship between variables, including direct path coefficient and indirect path coefficient. Direct path coefficient is to describe the influence of independent variable itself on dependent variable, while indirect path coefficient aims at analyzing the effects of independent variable on dependent variable by others. Path analysis is expressed by the measuring models in the following equations:

$$\begin{cases} a_1 + r_{12}a_2 + ... + r_{1p}a_p = r_{1y} \\ r_{21}a_1 + b_2 + ... + r_{2p}a_p = r_{2y} \\ r_{p1}a_1 + r_{p2}a_2 + ... + a_p = r_{py} \end{cases} \quad \text{(Eq. 5)}$$

Where a_i is the partial regression coefficient for variable i; r_{ij} is the correlation coefficient between x_i and x_j; r_{iy} is the correlation coefficient between x_i and y, $i, j = 1, 2, ..., p$.

As is shown in Equation (5), a_i is the direct effect of x_i on y, namely x_i is also the direct path coefficient for y; $r_{ij}a_j$ is the indirect effect of x_i on y by x_j ($x_i \leftrightarrow x_j \rightarrow y$), namely $r_{ij}a_j$ is also the indirect path coefficient for y by x_j. $r_{ij}a_j$ can be calculated so described as:

$$r_{iy} = a_i + \sum_{j=1} a_j r_{ij} \quad \text{(Eq. 6)}$$

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In path analysis, the coefficient of determination called R^2 indicates the effect of independent variables on dependent variable. If the coefficient of determination is significant, the path analysis is valid, otherwise it is meaningless. ${R_{(i)}}^2$ is calculated as follows:

$$R^2 = \sum_{i=1}^{p} R_i^2 + \sum_{i<j}^{p-1} R_{ij}^2 = \sum_{i=1}^{p} b_i r_{iy} \quad \text{(Eq. 7)}$$

Where R_i^2 is the coefficient of direct determination for effect of x_i on y, namely $R_i^2 = a_i^2$; R_{ij}^2 is the coefficient of indirect determination for effect of x_i on y by x_j;

Path analysis has been commonly applied to studying the direct and indirect effect of independent variables on dependent variable, but it is very difficult to identify which independent variable plays the most important role for dependent variable. So decision coefficient is introduced in this paper. Compared with the coefficient of determination, value of decision coefficient can order the potential combined effects of independent variable on dependent variable. The independent variable of the first order is the most critical decision one, but it does not mean that independent variable of last order has no effect. If decision coefficient is negative, it shows that independent variables have restrictive effect on dependent variable (Yuan et al., 2011). ${R_{(i)}}^2$ is calculated as follows:

$${R_{(i)}}^2 = a_i^2 + 2\sum_{j \neq i} a_i r_{ij} a_j = R_i^2 + \sum_{j \neq i} R_{ij}^2 \quad \text{(Eq. 8)}$$

Where R_i^2 is the coefficient of direct determination for effect of x_i on y; R_{ij}^2 is the coefficient of indirect determination for effect of x_i on y by x_j; a_i is the direct path coefficient for the direct effect of x_i on y; $r_{ij}a_j$ is the indirect path coefficient for the indirect effect of x_i on y by x_j.

In a word, the path analysis aims at analyzing the direct, indirect and comprehensive effect of independent variable on the dependent variable by the direct and indirect path coefficient and decision coefficient, based on building the regression equation.

Econometric model

Choice of influencing factors about water supply in Shenyang city

Although the relationship between forests and water is a complex issue, forest ES plays an important role in improving ecological environment of water resources, which are mainly attributed to water regulation, water purification and soil and water conservation. Water regulation is deemed that water resource is redistributed by

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maintaining or regulating hydrological flows and runoff recharging. Water purification aims at cleansing water quality. This is achieved through reduction of excessive concentrations of organic matter and filtering various types of pollutants in water bodies. Soil and water conservation is generally associated with prevention of soil erosion and with maintaining water resources. The surface cover, debris and tree roots are more effective in keeping the water. Moreover, the role of the upstream forests in regulating water flows, purifying water quality of the upper watershed and supplying water in downstream areas cannot be ignored. To analyze this effect under particular hydrological and ecological circumstance, water-related ecological system will be introduced in river basin. This system of water supply comprises two major activities: ecological production and stream flow/natural water (Matete and Hassan, 2006). Ecological production refers to transfer value of forest ES (X_1), and stream flow or natural water includes water quantity and water quality, consisting of stream flow and groundwater (X_2), chemical oxygen demand (COD) (X_3) and the standard-reaching rate of water quality (X_4) in this paper (*Table 7*).

Table 7. *Influencing factors of water supply in Shenyang city from 2010 to 2014*

Year	Transfer value of forest ES (10^9RMB)	stream flow and groundwater (10^9m^3)	COD (10^5m^3)	Standard-reaching rate of water quality (%)	Water supply (10^9m^3
2010	10.81	46.40	0.95	94.86	28.40
2011	10.84	45.44	0.94	95.89	28.43
2012	11.28	47.71	0.93	99.50	28.76
2013	11.60	47.71	0.92	97.64	28.98
2014	11.94	48.19	0.94	100.00	29.00

Table 8. *Correlation coefficient between water supply and influencing factors*

Variables	X_1	X_2	X_3	X_4	Y	Sig.
X_1	1.0000	0.8897	-0.4864	0.8383	0.9694	0.0064
X_2	0.8897	1.0000	-0.4915	0.8449	0.9076	0.0333
X_3	-0.4864	-0.4915	1.0000	-0.4695	-0.6769	0.2094
X_4	0.8383	0.8449	-0.4695	1.0000	0.8285	0.0830
Y	0.9694	0.9076	-0.6769	0.8285	1.0000	0.0001

Correlation analysis

Correlation analysis is often used to describe the degree of linear correlation among the variables. Although the methods of correlation analysis are various, many studies often rely on the correlation coefficient as an important indicator for measurement of linear correlation among the variables (Zhu et al., 2008). Pearson's correlation

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coefficient was applied to analyze the relationship between the influencing factors and water supply of Shenyang city (*Table 8*). The purpose of correlation analysis is to do some preliminary analysis on the relationship among variables and to prepare for building the model (Zhang et al., 2012).

As is shown in *Table 8*, correlation coefficient between X_i and Y showsthe ordering: $Px_1 > Px_2 > Px_4 > Px_3$. The results implies that water supply(Y) is significantly influenced by transfer value of forest ES(X_1). In addition, it indicates that it is negative correlation between chemical oxygen demand (COD) (X_3) and water supply (Y), and positive correlation between water supply (Y) and transfer value of forest ES (X_1), stream flow and groundwater (X_2) and standard-reaching rate of water quality (X_4).

Regression model

In order to facilitate analysis and explanation, the econometric model is built based on the chosen explanatory variables by means of multiple linear stepwise regressions. Specific calculations are completed by the SPSS17.0. The econometric model is intended to be constructed as follows:

$$Y = 0.497X_1 - 6.818X_3 + 29.486 \qquad \text{(Eq. 9)}$$

Where Y is the water supply in Shenyang city, X_1 and X_3 are the variables of transfer value of forest ES and chemical oxygen demand respectively. The result and test values of this are shown in *Table 9*.

Table 9. *The result and test values for influencing factors of water supply*

Variables	Coefficients	t	Sig.	Collinearity
Constant	29.485	18.612	0.003	-
Transfer value of forest ES	0.497	14.608	0.005	1.310
Chemical oxygen demand	-6.818	-4.687	0.043	1.310

Notes: R=0.997; R^2=0.995; F (sig.) =197.793(0.05); Durbin-Watson d=2.8335

As is shown from *Table 9*, only the variables, 'transfer value of forest ES' and 'chemical oxygen demand', have passed the F test and t test excluding the other variables- 'stream flow and groundwater' and 'standard-reaching rate of water quality'. The goodness-of-fit of this model is satisfactory.

Table 10. *Analysis of path correlation between water supply and influencing factors*

Variables	Direct effect	$\to X_1$	$\to X_3$	Indirect effect	Total effect	Decision coefficient
X_1	0.8385	—	0.1309	0.1309	0.9694	0.9226
X_3	-0.2691	-0.4079	—	-0.4079	-0.6770	0.2919

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The results of the model have further verified the previous inference from qualitative analysis. Transfer value of forest ESplays an important roleas an influencing factor which can regulate the recharging of groundwater aquifers and improve the function of the hydrologic cycle. However, stream flow and groundwater as the critical resource capturing and processing energy to provide water for Shenyang city is excluded in the model, it indicates that this variable has not mainly linear relationship. In another word, it cannot significantly increase water yield. Because several natural factors are alteringforest's role in influencing the availability of water resources (Bergkamp et al., 2003; Balkhair, 2016; Wu and Feng, 2016; Meng et al., 2016; Zhang, 2013), including other meteorological variables (precipitation and temperature). Chemical oxygen demand (COD) is regarded as a critical indicator to impair water quality. Because potential pollutants come from agricultural or industrial chemicals and domestic sewage, excessive concentrations of organic matter have greatly negativeeffect on drinking-water–supply. Adequate riparian forest buffer zones along watercourses can be maintained, which helps protect water and soils and even reduce pollutants flowing to rivers so as to decrease chemical oxygen demand (COD) and cleanse water quality. So the results show that the delivery of high-quality water can be ensured by the upstream forest, but the benefits of the upstream forest on increasing downstream water yield were generally misleading. This conclusionis the same as the results confirmed by FAO and CIFOR (2005).

Path analysis

Based on the above econometric model, this part is to explain the effects of transfer value of forest ES in the upper watershed on water supply in Shenyang city by the means of path analysis.

It can be seen from *Table 10* that the decision coefficients of 'transfer value of forest ES' and 'chemical oxygen demand (COD)' to water supply in Shenyang city are 0.9226 and 0.2919 respectively, which shows that the former affects water supply in Shenyang city more significantly than the latter. Besides, the former produces positively indirect influence on water supply, while the latter has negatively indirect influence.

Forest ES in the upper watershed provides valuable regulating services for drinking-water–supply of the downstream. It mitigates water pollution by preventing excessive concentrations of organic matter from directly reaching the sea and the nearby beaches.

When it rains, canopy layer intercept the precipitation, it is under the action of gravity that water flows into the soil carrying large amounts of nutrients can be absorbed, regulated and redistributed by forest ES functions, which produces the stream flow and groundwater. Meanwhile, in the cyclic process of hydrological and ecological circumstancevarious chemicals can be filtered in water bodies and chemical oxygen demand is gradually reduced to prevent pollutants from reaching the streams. When rivers are carriers of nutrients and pollutants, the reduction of sediment delivered to streams has a positive impact on water quality of the downstream and the livelihoods and health of downstream populations. So the transfer value of forest ES from the upper watershed not only regulate seasonal availability of water, but also cleanse water quality for Shenyang city.

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Conclusion

Undoubtedly, transfer value of forest ES undoubtedly has a significant impact on water supply in Shenyang city directly and indirectly. Main conclusions are as follows:

(1) Forest ES values are 111.61 billion RMB and 12.58 billion RMB respectively in the upstream and Shenyang city. With forest resources as the medium, transfer value have reached 56.57 billion RMB between the upstream and Shenyang city from 2010 to 2014, in addition to meet its own need for forest ES.

(2) Transfer value of forest ES has been identified as an important factor of water supply. According to the results of econometric models, it is largely correlated with each other. Therefore, transfer value of forest ES is not only an essential assurance for Shenyang city to share the balanced and sustainable availability of water resources, but also an effective guarantee to provide benefits to the urban populations in terms of high-quality drinking water.

(3) This results further carry out preliminary discussion about the influence of transfer value of forest ES on regulating water and cleansing water quality by the path analysis. It produces the positive externalities, which is beneficial to the stakeholders and greatly promotes formulation and implementation of ecological compensation policy and the coordinated and sustainable development among some districts.

Discussion

We have attempted to assess the transfer value of forest ES in the upper watershed, and have described its effect on water supply in river basin areas by the path analysis method. From this analysis it shows that there are some critical theoretical and empirical issues that remain to be resolved.

(1) According to the existing research results, forest ESV was used to statically evaluate by the market value method, production cost method and opportunity cost method, which only measure ESV in a certain region, but not calculate the spillover value of forest ES to other regions. In this paper, in addition to meet its own need for forest ES measured, the spillover value of forest ES can be still dynamically calculated from the upstream to the downstream in Hunhe River Basin with the help of Buffer and Intersect of ArcGIS9.3. The results are important proofs of payment for ecological services in the theory.

(2) The relation between transfer values of forest ES and water supply is very complex. At present, most of the research results focus on precipitation input, runoff output and the change of element content in the system cycle, but the interaction of each factor and a certain factor indirectly influenced by others may be ignored in forest hydrological process. The path analysis demonstrates that forest ESV from the upstream produces direct and indirect impacts on water resource from downstream in the river basin. Due to limited data, the impact of transfer value of forest ES on water resources is also affected by other factors, which needs further study in the future.

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(3) Forest ES are affected by many factors that are different in different regions, including light and heat, precipitation and site conditions. While these are beyond the scope of this paper, our analysis only can serve as a useful starting point, and these factors could be considered in further study.

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EFFECTS OF DRIP IRRIGATION SYSTEM FOR ENHANCING RICE (*ORYZA SATIVA* L.) YIELD UNDER SYSTEM OF RICE INTENSIFICATION MANAGEMENT

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Abstract. To increase both water productivity and rice production, research was carried out at the Central Institute of Agricultural Engineering, Bhopal, India, to evaluate the use of drip irrigation together with modifications in rice crop geometry and other cultural practices. Experiments were conducted with five alternative treatments: T_1: Conventional paddy cultivation under continuous flooding, T_2: System of Rice Intensification (SRI) methods which include alternate wetting and drying, T_3: SRI methods with drip-irrigation, the emitters being spaced at 20 cm, T_4: SRI methods with drip emitters spaced at 30 cm, and T_5: SRI methods with drip emitters spaced at 40 cm. Greatest plant height and root length were recorded with SRI under drip irrigation at 20 cm spacing (T3). Yield and yield-contributing parameters were also significantly higher with this T3 treatment as compared with conventional practice (TI): productive tillers/m^2, number of grains per panicle, average panicle length and weight, average grain yield (7 t/ha), and harvest index. Superior performance of the T3 treatment in terms of water productivity and water-energy productivity was also recorded. Among the drip-irrigated treatments, however, there was no statistically significant difference in grain yield between T3 and T4 treatments, indicating that drippers spaced at 30 cm could be recommended to lower the cost of the drip system installation.
Keywords: *water productivity, plant growth parameters, yield contributing parameters, water energy productivity, economics*

Introduction

Rice is the most important global food crop. Year-to-year fluctuations in its production have a direct bearing on food security and on price inflation in the world market. Given its continuing population growth, India should be producing 1.7 million tonnes of additional rice every year to ensure national food security (Dass and Chandra, 2013). Hence, there is a need to increase sustainably the yields and productivity of rice cultivation, doing this to the extent possible with reduced inputs and with less exploitation of natural resources to feed the increasing global population. This is a challenge for rice-growing countries around the world.

For producing rice at present with traditional irrigation techniques, large quantities of water are being used to flood paddy fields with standing water 2-5 cm deep at the different stages of crop growth. Studies have indicated that 3000-5000 liters of water are often used to produce 1 kg of rice (Satyanarayana et al., 2007), but this includes water applications which are clearly excessive. Water requirements for flooded rice production are currently considered to average a little over 1,400 liters per kg of rice(www.knowledgebank.irri.org/step-by-step-production/growth/water-management). This is about three times more than for growing wheat and maize (Riaz, 2001).

Water scarcity is a major challenge affecting rice production all around the globe. More than 80% of the fresh water resources in Asia are used for agriculture, of which

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about half of the total irrigation water is used for rice production (Dawe et al., 2003). Future rice production will depend on improvements in water use efficiency for growing rice crops. A large-scale evaluation in Indonesia with over 11,000 on-farm comparison trials conducted over six seasons reported a 78% increase in paddy rice yields with about 40% reduction in water and with lower costs of production when using the methods of the System of Rice Intensification (SRI), which include alternate wetting and drying of paddy fields rather than flooding as done with conventional practices (Sato and Uphoff, 2007). SRI methods have been validated in over 50 countries, and given the scarcity of water resources in so many countries and the evident yield advantages with SRI management, probably more than 10 million farmers are already using some or all of its recommended practices (http://sri.cals.cornell.edu).

Substantial water savings with higher paddy yields using SRI methods have been confirmed in a meta-analysis of 27 published studies across 8 countries, an average 35% reduction in irrigation water per hectare and a 22% reduction in total water (irrigation plus rainfall) (Jagannath et al., 2013). But these comparisons were all made with modified regimes of flood irrigation. Possibly there could be even greater savings of irrigation water and more gains in water productivity with employment of drip irrigation technology. And if such benefits are possible, how can the productivity of drip irrigation be optimized? These are important considerations for enhancing both the productivity and sustainability of irrigated rice cultivation, and it is these concerns that gave impetus to the research whose findings are reported here.

Materials and Methods

Study areas

The research work was carried out at the Central Institute of Agricultural Engineering, Bhopal. Soils of the experimental site are classified as heavy clay soils with clay content varying between 49.7 to 53.7 % and with the field capacity ranging from 28.5 to 31 %.Which was situated at north of Bhopal at 77º 24' 10" E, 23º 18' 35" N at an elevation of 495 m above mean sea level.

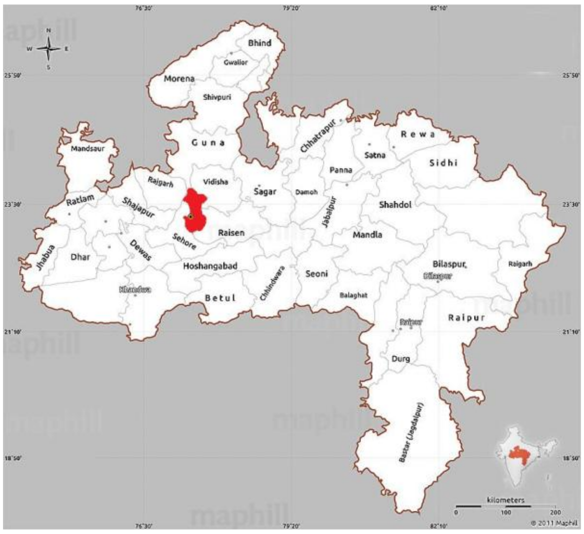

Figure 1. *The map of Madhya Pradesh State showing the Local Government Areas (red colour pointing to the study areas)*

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The climate of Bhopal is pleasant throughout the year. Generally the climate of the study area is hot and humid in nature. The annual rainfall in the region is about 1200 mm. Eighty percent of the total rainfall occurs during the month of June to September in almost every year which is popularly known as monsoon rainfall. The summer months from March to May are very hot and humid, and temperature often rises above 40ºC.

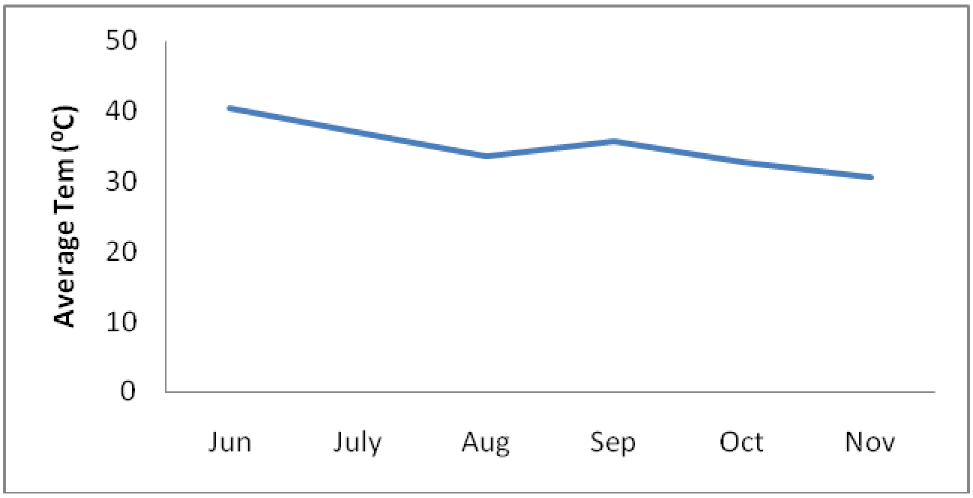

Figure 2. *Average temperature (ºC) of ten years*

Five treatments were considered in the study: T_1: Conventional practices with continuous flooding, T_2: System of Rice Intensification (SRI) crop and water management methods, T_3: SRI management with drip irrigation emitters spaced at 20 cm, T_4: SRI with drip emitters spaced at 30 cm and T_5: SRI with drip emitters spaced at 40 cm. Puddling was carried out in the T1 and T2 plots, but the T1 plots had continuous flooding, while T2 plots had alternate wetting and drying. Young seedlings 10 days old were transplanted manually in the T2, T3, T4 and T5 treatments at 30 x 30 cm spacing with single plants per hill and a seed rate of 10 kg ha^{-1}. Transplanting in T1 was carried out on the 30th day after seeding, and 3-4 plants per hill were transplanted at a spacing of 15 x 5 cm spacing, with a seed rate about 100 kg ha^{-1}. The dates of transplanting for all the treatments were the same. Each treatment plot was replicated four times, and each plot was surrounded by bunds 0.5 m wide in order to arrest any lateral seepage and nutrient diffusion from adjoining treatments.

Inorganic fertilizers were applied to all the plots with the recommended doses of 150 kg N, 100 kg P and 120 kg K per ha in the form of Urea, Di-Ammonium Phosphate (DAP) and Murate of Potash (MOP), respectively. Half of the recommended dose of nitrogen and all of the phosphorus and potash were applied at sowing, while the remaining N was applied at the time of tillering through broadcasting in the T1 and T2 treatments and through the drip irrigation system in T3, T4 and T5 treatments using water-soluble fertilizers. Rates and kinds of soil nutrient supplementation were thus not a variable in this experiment.

During the crop growing period, T1 treatment plots were under fully ponded conditions (to a depth of 4-5 cm) for a total of 34 days during the growing season, while T2 plots were under ponded conditions for a total of 25 days during their alternating periods of drying and wetting of the plots at six-day intervals. The drip-irrigated

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treatments T3, T4 and T5 were operated for 15 min, 23 min and 30 min, respectively, for a cumulative period of 60 days. Weeds were controlled by manual weeding after transplanting on the 20th, 40th, 60th and 80th days, so weed control was also not a variable evaluated in these trials even though mechanical, soil-aerating weeding is recommended as part of the recommended SRI protocol.

Monocrotophos (400 ml per acre) was applied for controlling stem borer at 25 DAS and Gandhi bug at 70 DAS in all treatments. Bacterial blight was controlled by the application of Streptocylin along with Copper Oxi-Chloride (6g+250g per acre) at 30-35 DAS in rice.

At the time of transplanting, five plants were randomly selected from each plot to be used for recording plant height, number of tillers, number of effective tillers, root length, panicle length, and panicle weight, number of grains per panicle, 1000-grain weight, grain yield, and straw yield. The data collected were analyzed by using Fisher's analysis of variance technique, and a Randomized Block Design (RBD) test with the confidence level set at 5% was used to compare the differences among treatment means (Steel et al., 1997).

Results and Discussion

Plant growth parameters

The influence of different treatments on rice crop growth parameters on the 60th day after transplanting is given in *Table 1*, and the actual condition of the crop is shown in *Plate 1*. The different irrigation practices along with associated crop management had a significant effect on plant height. The greatest average plant height was recorded with treatment T3 (76.45 cm) possibly due to optimizing plant population and geometry under SRI with drip emitters spaced at 20 cm which gave more even distribution of water to the plants. Similarly, the greatest number of tillers m^{-2} area was also recorded under T3 (240.75), followed by T4 (232.50) and T5 (223.50). The lowest number of tillers was recorded with T1 (217.25). However, chlorophyll values as measured by a SPAD meter did not significantly vary across the different treatments. Root length was also greatest under T3 (18.23 cm), followed by T4 (16.08 cm), while the lowest root length was recorded under T1 (14.10 cm).

Table 1. *Influence of growth parameters by different irrigation practices*

Treatments	Plant height at 60 DAT (cm)	Number of tillers at 60 DAT	SPAD Values	Root length (cm)
T1	73.45	217.25	44.81	14.10
T2	73.60	218.20	44.67	15.30
T3	76.45	240.75	45.12	18.23
T4	75.80	232.50	45.00	16.08
T5	74.70	223.50	44.85	15.50
SEM±	0.85	2.92	0.68	0.68
CD (p=0.05)	2.51	8.60	NS	2.01

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Conventional paddy (L), SRI (R)

Drip-irrigated paddy emitters spaced at 20 cm (L), 30 cm (M) and 40 cm (R) at 60 dayI (R)

Plate 1. *Experimental crops under different cultivation and irrigation practices at 60 days*

Intermittent irrigation creates favourable soil physical, chemical and biological properties that support plant growth under mostly aerobic soil conditions, encouraging deeper rooting depth and creating favourable micro-climates in the soil, which support abundance of micro-organisms and more availability of micro-nutrients. Better root systems provide good anchorage for the plants and sustain effective use of applied fertilizers by checking losses from leaching (Stoop et al., 2002). Optimum availability of soil moisture plays a key role in the processes of mineralization and solubilisation, affecting the availability and uptake of nutrients, and contributing to plant growth and yield (Venkateswarlu et al., 1997). Greater root capacity also supports a higher photosynthetic rate by supplying sufficient amounts of nutrients to the shoots, thereby enabling high productivity (Osaki et al., 1997). Sharda et al. (2016) observed in their study that root length and density were maximum in the drip irrigated crop than flood irrigated crop.

Yield-contributing parameters

It has been seen in many studies that flooding is not the most favourable condition for growing rice crops (Belder et al., 2004; Sahrawat, 2000; Gani, 2001; Borrel et al., 1997). The findings of the present study confirm that different irrigation methods and regimes have a significant influence on crop yield parameters. System of Rice Intensification methods adapted with drip irrigation emitters spaced 20 cm apart gave the highest number of productive tillers at maturity (264.75 m^{-2}), also the highest number of grains per panicle (161.75), longest panicle length (27.52 cm), and highest panicle weight (3.41 g), as seen in *Table 2*.

These findings are consistent with the results reported by Yang et.al. (2004) indicating that intermittent irrigation and maintaining moist, mostly aerobic soils not only enhances tillering but also the root system's development and functioning. However, 1000-grain weight and straw yield (t/ha) were not found to differ significantly among the five treatments. Treatment T3 also had a significant effect on grain yield and

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on the harvest index. SRI methods with drip at 20 cm spacing gave the highest grain yield, 7.07 t ha^{-1}, while conventional practices (T1) with flood irrigation, older seedlings, and greater plant density resulted in the lowest grain yield, 3.14 t ha^{-1}. Harvest index calculated for T3 was the highest (61.9), while that for T1 was the lowest (40.7), a very large difference.

Table 2. *Yield and yield-contributing characters influenced by different irrigation practices*

Treatments	Number of effective tillers (per m^2)	Number of grains per panicle	Panicle length (cm)	Panicle weight (g)	1000 grain weight (g)	Grain yield (t/ha)	Straw yield (t/ha)	Harvest index (%)
T1	222.25	133.50	25.67	2.78	32.30	3.14	4.30	40.73
T2	242.0	141.0	25.97	3.02	32.96	4.04	4.18	48.08
T3	264.75	161.75	27.52	3.41	33.51	7.07	4.60	61.93
T4	256.25	151.5	26.96	3.37	32.71	6.72	4.34	60.98
T5	253.0	149.0	25.87	3.16	32.59	5.95	4.43	57.72
SEM±	4.49	4.09	0.40	0.07	0.41	0.31	0.13	2.57
CD (p=0.05)	13.25	12.07	1.18	0.22	NS	0.90	0.39	7.59

It is worth noting that the 1st, 2nd, 5th and 7th sets of bar graphs in *Figure 3* all follow the same sequential pattern: T3 > T4 > T5 > T2 > T1. This pattern is seen throughout the data gathered and analyzed. Detailed measurements made of grain characteristics and milling quality (grain length, breadth, eccentricity, perimeter and milling efficiency) revealed no significant differences when the data were analyzed statistically (data not shown). However, there was a rather consistent rank-ordering of the results seen across all these measurements: T3 > T4 > T5 > T2 > T1.

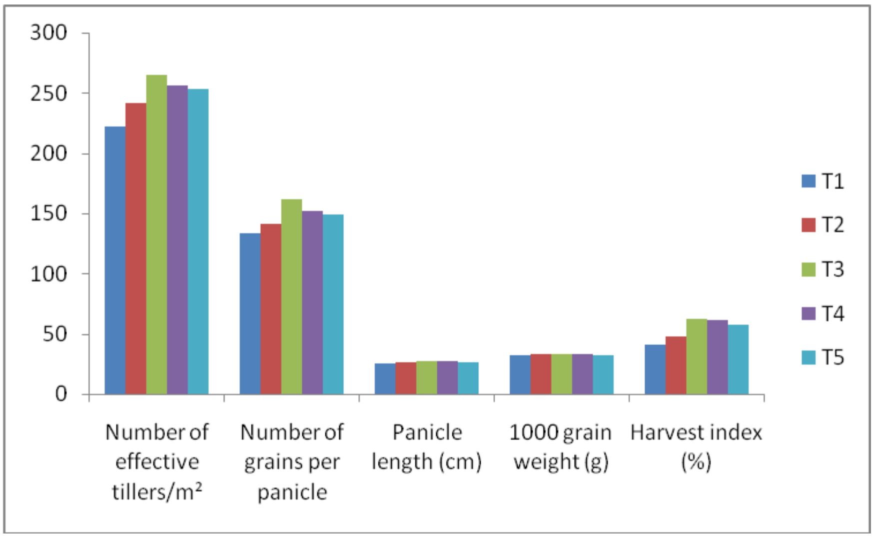

Figure 3. *Yield-contributing characteristics as influenced by different irrigation practices*

Water productivity and water-energy productivity

Efficient water management is key to higher paddy yields and greater net income with the System of Rice Intensification as this key input influences the effects of other

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inputs as well. Water productivity and water-energy productivities for the selected treatments were analyzed and are presented in *Figure 4*. Water productivity is the amount of grain yield obtained per unit of irrigation water supplied to the crop. Water-energy productivity is the amount of grain yield that is obtained from a unit of energy (kWh) consumed during the irrigation.

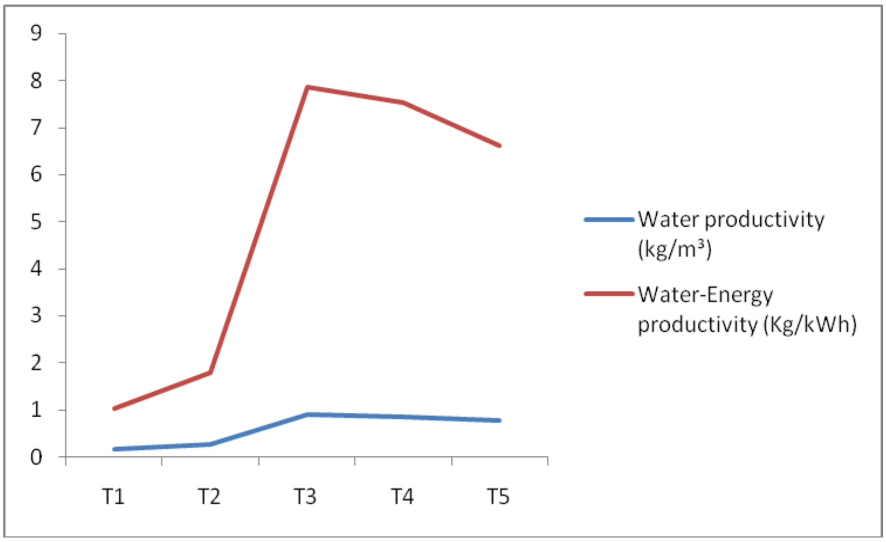

Figure 4. *Water productivity (kg of rice per m^3 of irrigation water) and water-energy productivity (kg of rice per kilowatt-hour of electricity) as influenced by the irrigation practices evaluated*

Water productivity was found highest under T3 (0.90 kg m^{-3}) followed by T4 (0.86 kg m^{-3}). The lowest water productivity was recorded under T1 (0.16 kg m^{-3}), with no difference between T4 and T3 in terms of water productivity. Similarly, the highest water-energy productivity of was observed under treatment T3 (7.85 kg kWh^{-1}), with the lowest productivity found for treatment T1 (1.02 kg kWh^{-1}). This difference was highly significant statistically. However, among the treatments T3, T4 and T5, the calculated differences in water productivity and water-energy productivity were not significant.

Economics of drip-irrigated paddy

In the present study, the economics of drip-irrigated paddy were compared with conventional paddy cultivation practices and with standard System of Rice Intensification. The annual cost of drip irrigation system was calculated with the assumption that the life of the physical infrastructure would be 10 years with 10 per cent annual depreciation. The cost components considered included seeds, fertilizer, labour, electricity charges per unit, farm yard manure, harvesting and threshing. Uniform cost values were considered for all the treatments depending upon the respective quantity of input in each treatment.

The findings of the study indicated that the highest net return ($ 2442/ha/year) and B:C (3.23) ratio can be obtained under SRI management with drip irrigation emitters spaced at 20 cm, followed by SRI with drip irrigation emitters spaced at 30 cm (*Table 3*). The lowest net return ($ 853/ha/year) and lowest Benifit: Cost (2.18) ratio was

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obtained with conventional practice of paddy cultivation. Based upon his research in Madagascar, Andrianaivo (2002) reported that optimal water use can enhance returns from System of Rice Intensification with enhanced labour productivity and far higher net income than traditional methods for the cultivation of rice. The findings of the present study are consistent with Andrianaivo's analysis and that of many other studies.

Table 3. *Economic and water productivity of paddy cultivation with drip irrigation systems over conventional system*

Treatments	Annual cost of cultivation ($/ha/year)	Gross monetary return ($/ha/year)	Net monetary return ($/ha/year)	Benifit:Cost ratio
T_1	1125	2170	1045	1.92
T_2	920	2769	1849	3.00
T_3	822	4782	3960	5.81
T_4	796	4562	3766	5.73
T_5	782	4041	3258	5.16

Assumptions: Revenue received is $ 667 per tonne of grain produced, and Rs. 17 per tonne for straw yield, based on prevailing market prices

Conclusions

The present study found consistent evidence that the adoption of drip irrigation combined with paddy transplanted and managed according to System of Rice Intensification practices offers substantial agronomic and economic advantages. Plant growth and yield-contributing parameters such as effective tillers, number of panicles m^{-2}, and grain yield were found to be significantly higher in drip-irrigated treatments. Apart from higher yield, water productivity and water-energy productivity were both higher in the case of drip-irrigated paddy cultivation with young single seedlings transplanted at 30 x 30 cm spacing with drip emitters spaced at 20 cm.

Among the drip-irrigated treatments, there was no significant difference in grain yield between the T3 and T4 treatments which indicate that spacing drippers at 30 cm can be recommended as the cost of installing a drip system would be relatively cheaper with more widely-spaced drippers. These results should be further tested with further experimentation and evaluation because the implications of this work could be rather far-reaching. The evidence assembled and analyzed here suggests that System of Rice Intensification crop management with drip irrigation is a promising adaptation for reducing the rice-crop's demand for water and energy, which are increasingly demanded and costly, while at the same time it raises grain yield. However, long-term, multi-location trials will be needed to arrive at percentages of water and energy saving that are achievable under varied and specific conditions.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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THE RESERVOIR RUNOFF FORECAST WITH ARTIFICIAL NEURAL NETWORK BASED ON ANT COLONY OPTIMIZATION

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Abstract. The results of reservoir water supply optimal dispatching depend to a great extent on the prediction of uncertain factors. Aiming at the problems of reservoir runoff forecast, an artificial neural network based on ant colony optimization is presented. The perturbation strategy is added in this new hybrid algorithm, the improvement ant colony optimization can combine the strong memory and associative ability of artificial neural network with positive feedback of ant colony optimization, and can overcome the shortcomings in the respect of lack of pheromone at initial stage and easiness of local optimization of ant colony optimization. It is applied to six reservoirs optimization water supply dispatching in the lower Luan he river. A practical example shows that this new hybrid algorithm is rational, reliable and high accuracy when it is used to solve the problem of reservoir runoff forecast, at the same time, the training precision is high. So, this new algorithm is applied to forecast the reservoir runoff.
Keywords: *back-propagation neural network; uncertain factors; water supply dispatching; perturbation strategy; Luan he river*

Introduction

The forecast of reservoir flow forecasts the hydro-logical regime in the future, which is mainly based on the hydro-logical and meteorological data and adopts the methodology of physical analysis and statistics. The traditional forecasting models mainly include genetic analysis and hydro-logical statistics. In recent years, with the development of computer technology, the methods of hydro-logical forecasting (Wan et al., 2016; Wei et al., 2013; Chang et al., 2013; Ahmad et al., 2017) mainly include chaotic analysis, grey system prediction, extension classified prediction method, artificial neural network method, wavelet analysis and the mixture of these methods. In this paper, we use the strong memory of neural network, positive feedback of association ability and ant colony algorithm, at the same time, we get rid of such problems as lacking information pheromone in the initial stage, getting trapped in a local optimum easily of ant colony algorithm in solving the model. In ant colony algorithm, we increase perturbation strategy, combine neural network and ant colony algorithm organically to propose a neural network algorithm based on ant colony optimization, forecasting reservoir runoff.

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Back-propagation neural network based on ant colony optimization

BP algorithm (Mohammad et al., 2013; He et al., 2014; Maryam et al., 2016) has the following advantages in theory, simpler network construction, strong memory and association ability, more stable state (Li et al., 2008; Wang and Zhang, 2010). But in the practical application of hydro-logic forecasting, there are some problems. The input of the new sample has an impact on the studied samples. We rely on experience to select the weights of the initial value and the number of neurons (Yi et al., 2013; Yang et al., 2016). Ant Colony Optimization or ACO (Qun et al., 2013; Kwang et al., 2014; Kleinkauf et al., 2015; Zhao et al., 2016; Vijay et al., 2016), is a probabilistic search algorithm with the advantages such as being parallel, positive feedback, strong robustness and excellent distributed computer system and so on.

Optimization model

In view of the above problems, the BP neural network is improved as follows: combining weights and parameters (A-BP) by using ACO and BP network, the network is trained, the training samples were normalized.

Basic principles of neural networks

Artificial neural network or ANN (Chen and Chang, 2009; Zhao et al., 2009), a nonlinear information parallel processing system (He et al., 2014; Shafaei and Kisi, 2014; Gharibreza, 2017) for simulating the structure and function of the neural network of the human brain, is composed of a large number of processing units (artificial neural networks) connected to each other and into the network, which has the characteristics of self-learning, self-adaptation, self-organization and so on (Khan et al., 2017). The neural network model was applied to the concept of energy function present a method for judging the stability of network in 1982, at the same time, it puts forward the concept of hidden unit. the weight adjusted error Back-Propagation algorithm for mufti- layer feed forward network was proposed In 1986, so that the research of artificial neural network is further studied. This kind of feed-forward network based on BP algorithm, generally called BP network, is one of the most popular neural networks currently. It can be used to approach the complex nonlinear function with high precision, which has the advantages such as simpler network construction, strong memory and association ability, more stable state.

The structure of BP neural network

BP network is generally composed of an input layer, an output layer, one or more of the hidden layer. There are a number of nodes in each layer. *Fig. 1* is the structure of BP network.

With an input layer of neurons, a hidden layer of neurons, an output layer of neurons. $x_1, x_2, \ldots, x_n$ are the input of the neural network, $y_1, y_2, \ldots, y_l$ are the output of the neural network? The information in the hidden layer can be used as input information in a non-linear way, and the output layer is transmitted to the output layer. The hidden layer is usually generated by the excitation function output information. Its excitation function is generally used Sigmund function. The information in the input layer must be transmitted to the hidden layer. The calculation process of BP network is composed of two parts, the forward calculation process and the reverse calculation

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process. Firstly, the input layer, hidden layer and output layer are calculated by layer by layer, then the output of the sample is get Secondly, the error between the expected sample output and the neural network calculation results is calculated by the error calculation formula. Finally, according to the error to adjust the network weight, the connection weights are corrected by the forward and backward, until the error meets the specified requirements.

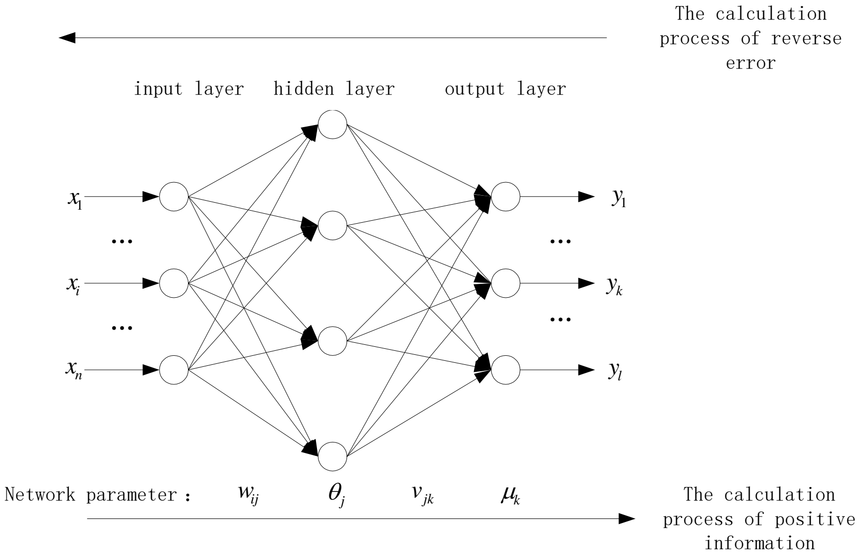

Figure 1. *The structure of BP Neural network*

(1) Random generation of initial threshold and weight

After determining the number of input layer neurons of BP neural network structure, the number of hidden layer neurons, the number of neurons in the output layer (n, m, 1). According to the basic principle of neural network, the parameters of neural network include the weights $w_{ij}(i = 1,2,\ldots, n; j = 1,2,\ldots, m)$ from the input layer *i* to the hidden layer unit *j*; activation threshold $\theta_j(j = 1,2,\ldots, m)$ of hidden layer units *j*; the connection weights $v_{jk}(j = 1,2,\ldots, m; k = 1,2,\ldots, l)$ of the hidden layer units *j* to the output layer units k; activation threshold $\mu_k(k = 1,2,\ldots, l)$ of output layer cell k .The initial weights of the network are generally randomly generated between [-1, 1].

(2) Output of Q training samples

Suppose there are training sample, the input $x_{iq}(i = 1,2,\ldots, n)$ of the first sample q $(q = 1,2,\ldots, Q)$ is transferred to the hidden layer. The output information h_{jq} of the hidden layer h_{jq} is obtained by the activation function $f_1(s)$.

$$h_{jq} = f_1(s_{jq}) = f_1\left(\sum_{i=1}^{n} w_{ij}x_{iq} - \theta_j\right) \qquad j = 1,2,\ldots, m; q = 1,2,\ldots, Q \quad \text{(Eq.1)}$$

Activation function generally selects Sigmoid function.

$$f(s) = \frac{1}{1 + e^{-x}} \quad \text{(Eq.2)}$$

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The output information h_{jq} of the hidden layer h_{jq} is transferred to the output layer, and the network output O_{kq} is obtained.

$$O_{kq} = f_2(r_{kq}) = f_2\left(\sum_{j=1}^{m} v_{jk} h_{jq} - \mu_k\right) \qquad k = 1,2,\ldots,l;\ q = 1,2,\ldots,Q \quad \text{(Eq.3)}$$

(3) Error calculation

There are usually some errors between the expected value y_{kq} and the output O_{kq} of the neural network. The error function $E(q)$ of the sample *Q* can be expressed as:

$$E_q = \frac{1}{2q}\sum_{q=1}^{Q}\sum_{k=1}^{l}\left(O_{kq} - y_{kq}\right)^2 \qquad k = 1,2,\ldots,l;\ q = 1,2,\ldots,Q \quad \text{(Eq.4)}$$

If the error meets certain accuracy requirements, the network learning ends, otherwise the network weights and thresholds are adjusted according to the error.

(4) To adjust the weights and threshold

The number of neurons in the neural network has been determined; it can reduce the error through the adjustment of threshold value and weight to have achieved the goal of improving the accuracy of calculation. w can be modified, Because the error function E_q changes along with the negative gradient w, so the correction value is Δw.

$$\Delta w(t+1) = w(t+1) - w(t) = -\eta \frac{\partial E_q}{\partial w} \quad \text{(Eq.5)}$$

In the formula, η is the learning rate, the range is from 0 to 1. w is certain weight or threshold.

Under normal circumstances, if η becomes larger, although the convergence of the network is fast. There will be oscillations; if η becomes smaller, the convergence of the network becomes slower. In order to avoid this situation, we can by inertia factor α, its values range from 0 to 1, which is analysed by (5).

$$\Delta w(t+1) = -\eta \frac{\partial E_q}{\partial w} + \alpha \Delta w(t) \quad \text{(Eq.6)}$$

For hidden layer:

$$\frac{\partial E_q}{\partial \theta_k} = \delta'_{jq} = f_1'(s_{jq}) \cdot \sum_{k=1}^{l} \delta_{kq} v_{jk}, \qquad \frac{\partial E_q}{\partial w_{ij}} = -\delta'_{jq} x_{iq} \quad \text{(Eq.7)}$$

Therefore, from the input layer to the hidden layer weights, the hidden layer threshold of the modified formula is:

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$$\Delta w_{ij}(t+1) = \eta\delta'_{jq}x_{iq} + \alpha\Delta w_{ij}(t) \quad \text{(Eq.8)}$$

$$\Delta\theta_j(t+1) = -\eta\delta'_{jq} + \alpha\Delta\theta_j(t) \quad \text{(Eq.9)}$$

For output layer neurons:

$$\frac{\partial E_q}{\partial \mu_k} = \delta_{kq} = (y_{kq} - O_{kq})\cdot f'_2(r_{kq}), \qquad \frac{\partial E_q}{\partial v_{jk}} = -\delta_{kq}h_{jq} \quad \text{(Eq.10)}$$

Therefore, the correction formula from the hidden layer to the weight of the output layer and the threshold of output layer is:

$$\Delta v_{jk}(t+1) = \eta\delta_{kq}h_{jq} + \alpha\Delta v_{jk}(t) \quad \text{(Eq.11)}$$

$$\Delta\mu_k(t+1) = -\eta\delta_{kq} + \alpha\Delta\mu_k(t) \quad \text{(Eq.12)}$$

After adjusting the weights, if the error does not meet the accuracy requirements, the procedure (2) is transferred to calculation, until the error meets the specified accuracy requirements.

The basic principle of ant colony optimization algorithm

Ant Colony Optimization or ACO, an optimization algorithm (Blum and Dorigo, 2004; Du et al., 2014) based on ant foraging principle, has the advantages (Deng and Peng, 2010; Yuan and Qu, 2013) such as parallel, positive and negative feedback, strong robustness and excellent distributed computer system. In initial time, the pheromone equals on all paths, the pheromone trail intensity $\tau_{ij}(0) = C_0$ (C_0 is a constant) from the nest i to the food source j. In the course of the movement, the ant $k(k = 1,2,\ldots, m)$ decides the direction of the transfer according to the amount of information on each path. At the moment of t, the transition probability $P_{ij}^k(t)$ of the ant k in the path i and the path j is:

$$P_{ij}^k(t) = \begin{cases} \dfrac{\tau_{ij}^{\alpha}(t)\eta_{ij}^{\beta}(t)}{\sum\limits_{s\in allowed_k}\tau_{is}^{\alpha}(t)\eta_{is}^{\beta}(t)}, & j \in allowed_k \\ 0, otherwise \end{cases} \quad \text{(Eq.13)}$$

In the formula, $allowed_k = \{0,1,\ldots, n-1\}$ is the next step to allow the ant k to choose the target; τ_{ij} is pheromone trail strength of edges (i, j); η_{ij} is heuristic factor for edge (i, j), P_{ij}^k is transition probability for the ant k , α, β are two parameters. The relative importance of the information and heuristic information accumulated during the process of the ants in the selection of ants reflected respectively. The amount of pheromone on each path is adjusted according to the following formula.

$$\tau_{ij}(t+1) = (1-\rho)\tau_{ij}(t) + \rho\Delta\tau_{ij}(t,t+1) \quad \text{(Eq.14)}$$

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$$\Delta\tau_{ij}(t,t+1)=\sum_{k=1}^{m}\Delta\tau_{ij}^{k}(t,t+1) \tag{Eq.15}$$

In the formula, $\Delta\tau_{ij}^{k}(t,t+1)$ is the pheromone of the ant k to stay on the path (i,j) at all times of $(t,t+1)$. The value depends on the merits and virtues of the ants. The shorter the path is, the more pheromone releases. $\Delta\tau_{ij}(t,t+1)$ is increment of the pheromone amount of the loop path (i,j). ρ is attenuation coefficient of pheromone. Usually, we set $\rho < 1$ to avoid an unlimited increase in the amount of path on the path.

In this paper, we will use the random perturbation strategy to prevent the stagnation of ant colony algorithm. The random selection probability needs to adjust dynamically. Approximate optimal solutions are obtained. The calculation time is shortened and the calculation efficiency is improved.

The ants' choices of their paths are random. Generally, they choose the path of transition probability, but the optimal path is not always selected, which results in a subsequent search for stagnation. Because the current optimal path information is more than the fact that doesn't find the optimal path pheromone number, with the increase of the number of iterations, the actual optimal path is less and less, so the probability of choosing this path becomes smaller and smaller. Considering the stagnation of the algorithm as well as based on the characteristics of the above ants in the choice of path at the same time, "disturbance factor" will be added to interfere with the ant selection path. The pheromone is not the most path to a certain probability of random selected, this path may be the best. At the same time, the probability of random selection in evolutionary computation which needs to be adjusted dynamically to increase the diversity of the selected paths, but the probability of the maximum path of the pheromone is calculated separately to prevent leakage of the optimal paths. The transition probability of the perturbation strategy can be formulated as follows:

$$C_{ij}^{k}=\begin{cases}\dfrac{(\tau_{ij}\eta_{ij})^{\gamma}}{\sum\limits_{s\in allowed_k}\tau_{is}^{\alpha}(t)\eta_{is}^{\beta}(t)} & \tau_{ij}\in\max\{\tau_{is}\}, s\in allowed_k\\ \dfrac{(\tau_{ij})^{\alpha}\cdot\eta_{ij}}{\sum\limits_{s\in allowed_k}\tau_{is}^{\alpha}(t)\eta_{is}^{\beta}(t)} & \tau_{ij}=\tau_{is}-\max\{\tau_{is}\}, p\le p_m, s\in allowed_k\\ \dfrac{\tau_{ij}\cdot(\eta_{ij})^{\beta}}{\sum\limits_{s\in allowed_k}\tau_{is}^{\alpha}(t)\eta_{is}^{\beta}(t)} & \tau_{ij}=\tau_{is}-\max\{\tau_{is}\}, p> p_m, s\in allowed_k\\ 0 & otherwise\end{cases} \tag{Eq.16}$$

In the formula, γ is a reverse exponential perturbation factor; $p_m\in(0,1)$ is random mutation rate; p abides to the uniform distribution of random variables. The formula indicates that ants in an iterative process can choose a number of paths. The formula (Eq.13) is used to calculate the transition probability on the path of the pheromone. In a random choice, we can calculate the transition probability on the alternative paths, the perturbation strategy is a combination of random selection and deterministic selection. The random selection makes the choice of the path and the calculation of the strong randomness, deterministic choice refers to the path that the ant will choose the most transition probability.

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Methods solution

The training samples were normalized

Because the input of the physical quantity is not the same, so the difference is very big, so the input data should be normalized before calculating to convert it from0 to 1 (logarithmic S curve). Normalized change is according to the following formula:

$$T = T_{\min} + \frac{T_{\max} - T_{\min}}{X_{\max} - X_{\min}}\left(X - X_{\min}\right) \quad \text{(Eq.17)}$$

In the formula, X is original input data. X_{max}, X_{min} are the maximum and minimum values respectively. T is transformed data, T_{max}, T_{min} are the maximum and minimum values settled respectively. T_{max} usually takes the rang from 0.8 to 0.9, T_{min} is 1- T_{max}.

Ant colony algorithm (ACO) to optimize the BP network

Firstly, ant colony algorithm is used to optimize the initial weights and network structure of BP neural network. The hydrological forecasting model is established. The network training and fitting experimental data should be gotten. Then the algorithm is applied to train the network samples so that the network output error is minimized. The effective improvement of BP neural network can easily fall into the local minimum value and the convergence speed is slow and all so defects. Suppose there are m parameters to be optimized of BP neural network, the parameters are arranged in order: $p_1, p_2, \ldots\ldots p_m$. According to any parameter, initialization N any nonzero value, the set I_{pi} is constituted. Suppose the ant number is S, all ants search for food by randomly selected element from first set, then return to the nest after finding food. Repeat this step, until all ants collect the same route, the optimum solution is obtained of this network. The specific calculation steps are as follows:

Step1: The BP neural network model is established, including the number of network layers, the number of nodes, the range and the sample size to be optimized.

Step2: Initialize ant colony. The parameters are uniformly dispersed. Initialization the path that according to discretize data. Then the complete path is established. The Pheromone trail intensity is $\tau_{ij}(0) = C_0 (C_0 is \text{ constant})$. The combination of discrete points of each parameter is defined as the path of ants that is a solution to the problem.

Step3: Cyclic iteration of ant colony algorithm. At the end of each iteration, generate random numbers q of the range from 0 to 1, make a comparison of explored relative importance threshold value $q_0 (0 \le q_0 \le 1)$ of the new path with the prior knowledge. If $q \le q_0$, the parameters of each weight are randomly variant according to the formula (Eq.16), random mutation is for new weight discrete points to get added to the collection S. If $q \ge q_0$, select the weight according to the formula (Eq.13).

Step4: All ants set out from set I_{pi}, follow the path rule to find element in order, finally the food source is found. The rule of route choice is: All ants *k (1,2,……M)* arbitrary choose j^{th} with certain probability. The probability formula:

$$\Pr ob(t_j^k(I_{pi})) = (t_j(I_{pi})) / \sum_{u=1}^{N} t_u(I_{pi}) \quad \text{(Eq.18)}$$

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Step5: When all the ants are *completed*, we input training sample. According to the formula (Eq.14) (Eq.15), the weight parameters were updated.

Step6: A set of the best weights that find by the ant colony algorithm are used as the initial weights of the BP algorithm. The error between the network output and the actual output is calculated. And the error is propagated from the output layer to the input layer, adjust weights, if the error reaches a predetermined precision or satisfy the maximum iterations number T, the algorithm is over. Otherwise, re-select ant colony to Step2.

The flowchart of an artificial neural network based on ant colony optimization is described in *Figure 2*.

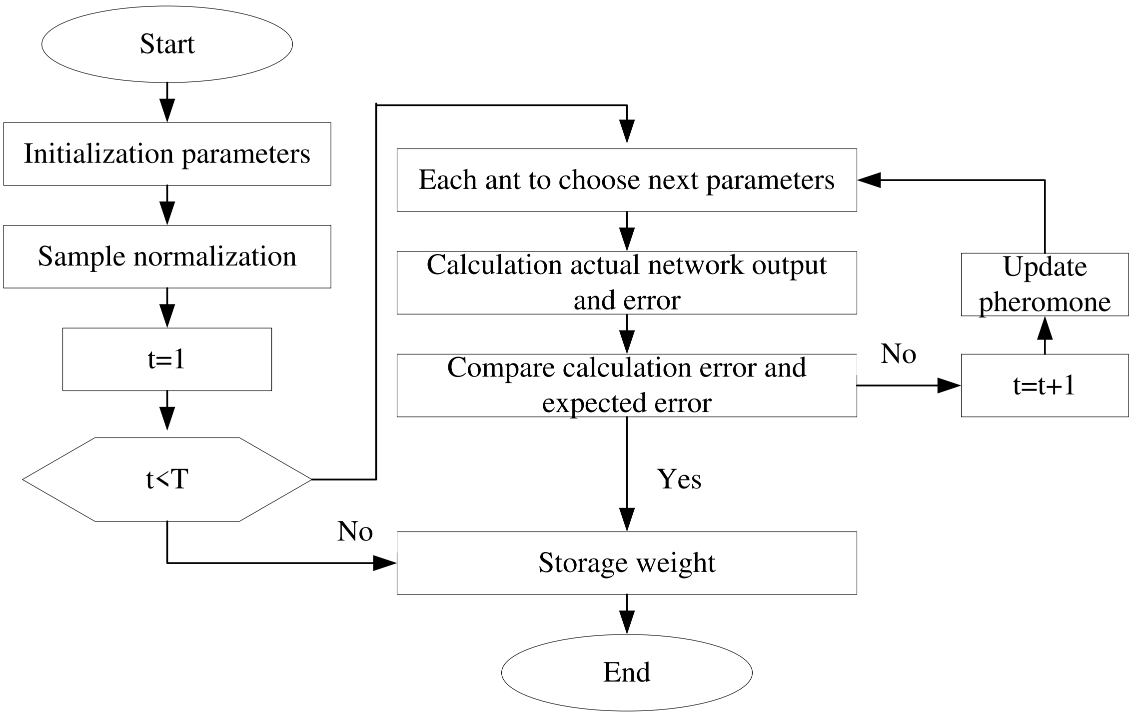

Figure 2. *The flowchart of an artificial neural network based on ant colony optimization*

Case study

The LuanHe River is rich in water resources in Northern China. The total precipitation ranges between $129\times10^8m^3$ and $12.7\times10^8m^3$, and has uneven distribution of inter-annual variations, with characteristic of a typical semi-arid and semi-humid region. The lower LuanHe River is the most important source that supply water to TianJin and TangShan which face severe challenge of the imbalance between supply and demand of water resource the situation is aggravated day by day. Five reservoirs have been constructed and are delivering an annual flow of 2873 million cubic meter water into the cities. The annual water deficiencies are 1025 million cubic meters. The flow supplies water for agricultural, industrial and municipal uses.

Each reservoir’s characteristics are as follows:

PanJiaKou reservoir is a carryover storage reservoir with total storage capacity is $29.3\times10^8m^3$, and its regulating the function is perfect; DaHeiTing reservoir is located in the PanJiaKou reservoir downstream, which is a annual regulation reservoir, and the

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total storage capacity is $3.37\times10^8m^3$; YuQiao reservoir is a larger-scale reservoir in TianJin, and total storage capacity is $15.59\times10^8m^3$; QiuZhuang reservoir which is an intermediate regulation reservoir and Taolinkou reservoir belong to QinHuangDao. To augment the water supply in the basin and to keep up with the increasing demand, inter-basin transfers have been implemented, by bring them into full play in a multiply connected reservoirs. The main project characteristic indexes of each water supply reservoir are described in *Table 1*.

Table 1. *Main project characteristic indexes of each water supply reservoir (Unit: 10^8m^3)*

Reservoirs		Dead storage	Active storage	Total storage	Water supply task
Donor reservoirs	PanJiaKou	3.31	19.50	29.30	No direct user, supply water to recipient reservoirs
	DaHeiTing	1.13	2.07	4.73	
Recipient reservoirs	YuQiao	0.36	3.85	15.59	Supply water to Tianjin of industry and domestic life
	QiuZhuang	0.008	0.65	2.04	Supply water to Tangshan of industry and domestic life
	Taolinkou	0.51	7.09	8.59	Supply water to Qinhuangdao of industry, domestic life and agriculture of lower LuanHe river

Results and discussion

Neural network model based on ant colony optimization makes a prediction of the runoff in the lower reaches of the Lutheran River Reservoir. Select Panjiakou Reservoir, Daheiting and Taolinkou Reservoir respectively (Given the limited space available, prediction of bridge reservoir, the results of Qiuzhuang reservoir and Yuqiao reservoir are not listed.) The research object is the data of inflow runoff in the 26 year. January in 1975 to December in 1995 as a training sample and regard data for network fitting test of 5 years from January in 1996 to December in 2000 as network fitting test (Ghosh and Dash, 2017).

Design guidelines for ACO-BP neural networks: The data is first trained by ACO algorithm, after training is completed, then the neural network is trained by the BP algorithm, the learning rate of BP algorithm is as small as possible so as to perform fine search behavior, the number of training of BP algorithm as much as possible not to exceed the average absolute error, the maximum relative error and mean square difference of 1000 times, because most of the global search work has been completed by the ACO algorithm, the BP algorithm only needs to perform a more detailed local search of the search's finishing work. BP neural network training

Parameters set as follows: the maximum number of training to take 800, learning efficiency to take 0.1, learning output error target to take 0.001. The ant colony algorithm is initialized as follows: the number of ants is 50, the important factor of pheromone is 1, the importance factor of the heuristic function is 5, the pheromone factor is 0.1, and the maximum number of iterations is 200, as shown in *Figs. 3-8*.

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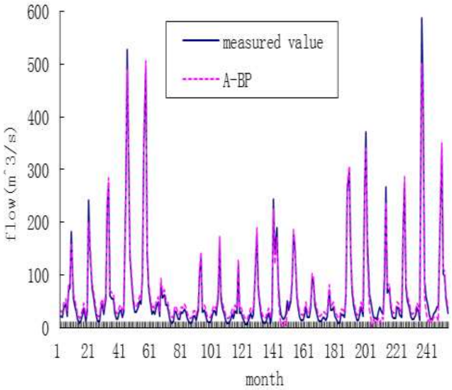

Figure 3. *The comparison figure between measured and trained values of monthly inflow of PanJiaKou (January 1975 - December 1995)*

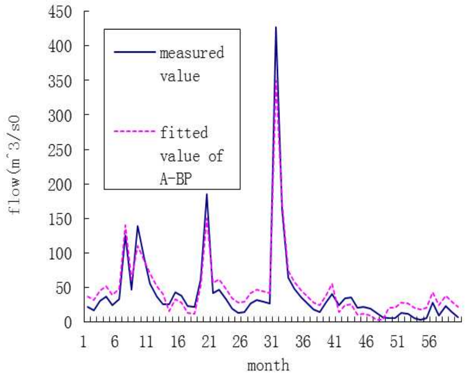

Figure 4. *The comparison figure between measured the fitted values of monthly inflow reservoir of PanJiaKou reservoir (January 1996 –2000)*

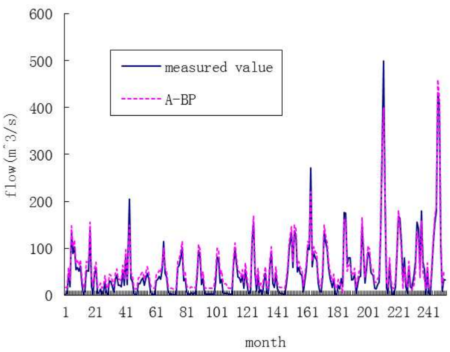

Figure 5. *The comparison figure between measured and trained values of monthly inflow of DaHeiTing (January 1975 - December 1995)*

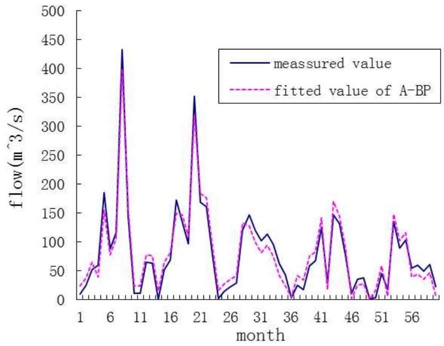

Figure 6. *The comparison figure between measured the fitted values of monthly inflow reservoir of DaHeiTing reservoir(January 1996 –2000)*

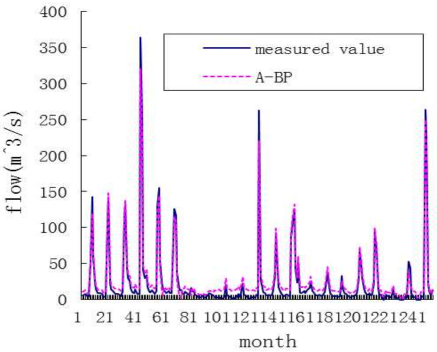

Figure 7. *The comparison figure between measured and trained values of monthly inflow of Taolinkou of Taolinkou reservoir(January 1996 –2000)*

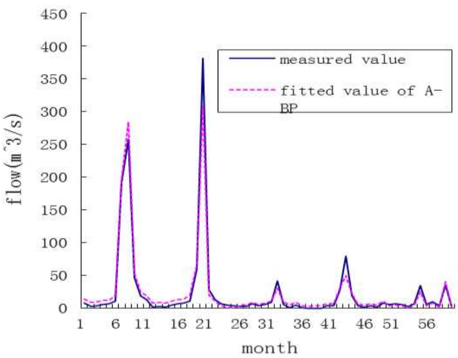

Figure 8. *The comparison figure between measured the fitted values of monthly inflow reservoir(January 1975 - December 1995)*

The Panjiakou Reservoir from the year 2001 to 2005 of annual runoff were predicted and compared with the actual value. The forecast effect is shown in *Fig. 8*.

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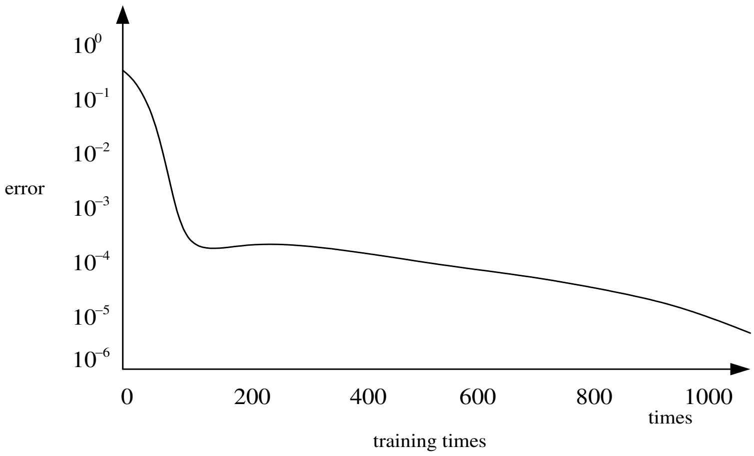

Figure 8. *The figure of error changing about network model trained values*

The analysis of *Figure 8* shows, the BP neural network model based on ant colony optimization is reasonable and reliable.it can make a prediction of reservoir runoff; According to the result of calculation Formula (4), the error between the fitting results and the measured values is compared within 10%. It shows that the training accuracy is higher and can meet the demand, so the runoff of the reservoir can be predicted.

In order to explain the prediction effect of different algorithms, the average absolute error (AAE), maximum relative error(MRE) and root mean square (RMS) are proposed.

$$AAE = \frac{1}{n}\sum_{k=1}^{n}\left|x_k - x^{'}\right| \quad \text{(Eq.19)}$$

$$MRE = \max\left|x_k - x_k^{'}\right| \quad \text{(Eq.20)}$$

$$RMS = \sqrt{\frac{\sum_{k=1}^{n}\left(x_k - x_k^{'}\right)^2}{n}} \quad \text{(Eq.21)}$$

In the formula, x_k is the network output value; $x_k^{'}$ is the true value; n is the total number of sample. The error comparison of several algorithms is described in *Table 2* with Panjiakou reservoir.

Table 2. *The error comparison of several algorithms with Panjiakou reservoir*

Difference algorithms	*AAE*	*MRE*	*RMS*
BP	6.24	9.62	7.63
PSO-BP	5.38	8.35	6.32
ACO-BP	4.25	7.01	5.24

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Through the comparison of error indicators which include average absolute error, maximum relative error and root mean square difference, etc, we can see from *Table 2*, the optimized BP neural network algorithm based on ant colony algorithm proposed in this paper has better fit performance and predictability, mainly because the PSO algorithm in the context of the search cannot easily find the local optimization, and easy to fall into them, so the accuracy of its operation is not very satisfactory. In the search process, it will find the optimal value, but there is no guarantee that this is the global optimal, the ant colony algorithm is relatively good in this respect. Thus, the optimized BP neural network algorithm based on ant colony algorithm improves the accuracy of hydrological prediction to reduce the error.

Conclusions

In this paper, the ant colony optimization algorithm is introduced into the neural network. Random disturbance strategies are added. In this paper, we make full use of the strong memory and associative ability of neural network as well as the characteristics of state stability and the local search ability of ant colony algorithm. At the same time, we get rid of such problems as lacking information pheromone in the initial stage, getting trapped in a local optimum easily of ant colony algorithm in solving the model, improving the accuracy of calculation. The algorithm is applied to the prediction of runoff in reservoir. The error between the fitting results and the measured values is compared to get the result within 10%, which is able to meet the requirements. In this paper, the neural network model based on ant colony optimization is reasonable and reliable, which can predict reservoir runoff, training accuracy is higher, so we can predict the runoff of the reservoir. In spite of this, the forecast of uncertain factors such as reservoir runoff are uncertain, but in the actual operation, water and water has great randomness, so we need further stud the real-time forecasting of reservoir runoff.

Acknowledgements. This study was supported by the National Key Research and Development Program of China (2016YFC0401401), Major Research Plan of the National Natural Science Foundation of China (91547209), the National Natural Science Foundation of People's Republic of China (51509089, 51379078, 51409103, 51579101), the Science-tech Innovation Talents in University of Henan Province (15HASTIT044).

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PROTECTIVE ROLE OF EXOGENOUS NITRIC OXIDE AGAINST ZINC TOXICITY IN *PLANTAGO MAJOR* L.

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Abstract. Zinc is one of the heavy metal in plants that causes toxicity at high concentration via producing Reactive oxygen species. Nitric oxide can protect cells from oxidative stress produced by reactive oxygen Species. Effect of different concentrations of Zn (0, 100, 300 and 500 μM) and interaction with sodium nitroprusside (SNP, a donor of NO) (100 and 200 μM) were studied on growth parameter (total dry Weight) and some physiological factors (chlorophyll a, chlorophyll b, hydrogen peroxide and malondialdehyde contents), antioxidant enzymes (catalase, peroxidase, ascorbate peroxidase and Superoxide dismutase) in *Plantago major* L. Excess Zn reduced dry weight and chlorophyll content, resulting a decrease in photosynthesis. Zn stress induced the production of hydrogen peroxide (H_2O_2), leading to malondialdehyde (MDA) accumulation. Furthermore, it was found that the activities of antioxidant enzymes in Zn-treated plants such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) were decreased but peroxidase (POD) and catalase (CAT) were increased. The use of NO especially in low concentration reversed Zn-induced negative effects whereas high concentration of NO had no obvious alleviating effect on Zn toxicity in *Plantago major* L. In particular, application of 100 μM SNP could mitigate Zn stress, as a defense mechanism of the plant against Zn toxicity.
Keywords: *heavy metal stress, sodium nitroprusside, antioxidative enzymes, ROS, hydrogen peroxide*

Introduction

Zinc is an essential nutrient element for plants, as it is the component of many proteins like Zn finger-containing transcription factors, carbonic anhydrase, Cu/Zn superoxide dismutase and Zn metalloproteases (Broadley et al., 2007). Many of these proteins have critical roles in the uptake and transport of Zn, transcriptional regulation, RNA binding, regulation of apoptosis and protein–protein interactions (Ciftci-Yilmaz and Mittler, 2008). Zn toxicity can result in nutrient imbalance and induce the generation of excess reactive Oxygen species (ROS) (Wang et al., 2009). The excessive formation of ROS can oxidize various cellular components, resulting in lipid peroxidation, membrane leakage, and enzyme inactivation, which can finally lead to oxidative injury and alteration in the cell structure (Romero-Puertas et al., 2007).

Plants have many detoxification and tolerance mechanisms that mitigate and repair the ROS damages. One of these mechanisms includes antioxidant systems, which help them, survive in the altered environment. Antioxidant enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), Catalase (CAT) and guaiacol peroxidase (POD), as well as

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antioxidant concentrations, like ascorbic acid, are activated by plants to alleviate oxidative stress (Tewari et al., 2008).

NO is an essential signaling molecule which modulates plant resistance to various abiotic stresses (Yu et al., 2014). NO is a highly reactive, membrane-permeable free radical that has recently emerged as a very important signaling molecule and antioxidant which triggers several types of redox-regulated (defense-related) gene expressions, directly or indirectly, to establish plant stress tolerance (Sung and Hong, 2010). The application of an NO donor, SNP, confers tolerance to various abiotic stresses in plants by enhancing their antioxidant defense system (Xu et al., 2010).

Several lines of study have shown that the protective effect of NO against abiotic stress is closely related to the NO-mediated reduction of ROS in plants (Hasanuzzaman et al., 2010). Exogenous application of SNP enhances plant tolerance to heavy metals (Oliviera et al., 2016) and oxidative stress (Esim and Atici, 2013). A recent report showed that NO was associated with long-term Zn tolerance in *Solanum nigrum* (Xu et al., 2010). NO involved on detoxification of H_2O_2 via modulation of antioxidant enzymes such as catalase (CAT),peroxidase (POD) and ascorbate peroxidase (AXP) and keeping of cell redox couple and non-protein antioxidant including thiol, ascorbate (Tewari et al., 2008). It also has been evidenced that NO stimulated the biosynthetic pathway of glutathione (GSH) in plant cells with an enhanced tolerance against oxidative stress (Xiong et al., 2010).

Plantago major is a medicinal herb with potent antioxidant effect .It belongs to the family of Plantaginaceae and is widely found in Europe and Asia in which Malaysia is one of the countries of its Habitat (Beara et al., 2009).

Nevertheless, the influence of exogenous NO on Zn stress tolerance in *Plantago major* plants is not yet fully understood. The present study was carried out to assess the impact of exogenous NO on reducing Zn toxicity in *Plantago major* L.

Materials and Methods

Plant material and culture conditions

Plantago major L. seeds were sterilized with 5% sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated on moist filter paper in the dark at 27°C for one week. Then, seedlings of uniform size were transferred to plastic pots filled with perlite (2 plants per pot) and irrigated by distilled water for 10 days. The seedlings were then nourished with half-strange Hoagland solution for three weeks, during 3 leaves stage irrigation was continued with half-strange Hoagland solutions containing different concentrations of ZnSO4 (0, 100, 300 and 500 μM) and sodium nitroproside (0, 100 and 200 μM) alone and together for two weeks. The experiment was carried out under a controlled environment, day/night temperature of 27/18°C and 65 ± 5% relative humidity. For the estimation of plant dry matter content, the plants were dried at 80°C for 48 h, to give a constant weight.

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Determination of photosynthetic pigments

The photosynthetic pigments (chlorophyll a and b) was determined as per the method of Lichtenthaler (1987). Chlorophyll of experimental plant was extracted with 80% acetone and centrifuged. Supernatant was taken and optical density was measured at 663 nm, 645nm. The chlorophyll a and b was calculated by Eq.1 and 2.

$$\text{Chlorophyll a } (\mu g/ml) = 12.21 \times A663 - 2.81 \times A645 \quad \text{(Eq. 1)}$$

$$\text{Chlorophyll b } (\mu g/ml) = 20.13 \times A645 - 5.03 \times A663 \quad \text{(Eq. 2)}$$

where A is the observed OD.

Determination of lipid peroxidation

Lipid peroxidation was determined by measuring MDA, a major thiobarbituric acid reactive species (TBARS), and product of lipid peroxidation (Heath and Packer 1968). Samples (0.2 gr) are ground in 3 mL of trichloroacetic acid (0.1%, w/v). The homogenate was centrifuged at 10,000 g for 10 min and 1 mL of the supernatant fraction was mixed with 4 mL of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA). The mixture was heated at 95°C for 30 min, chilled on ice, and then centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

Estimation of hydrogen peroxide (H_2O_2)

H_2O_2 concentration from leaf samples was measured according to the procedure of Velikova et al. (2000). Fresh leaf tissue (0.5 gr) was homogenized with 5 mL of 0.1% (w/v) (TCA) in a pre-chilled mortar and pestle and the homogenate was then centrifuged at 12,000 g for 15 min. To 0.5 mL of the Supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of potassium iodide (1 M) were added. The mixture was vortexed and its absorbance was read spectrophotometrically at 390 nm.

Determination of antioxidant enzymes

For extraction of antioxidative enzymes, leaves and roots were homogenized with 50 mM Na2HPO4-NaH2PO4 buffer (pH 7.8) containing 0.2 mM ethylene diamine tetra acetic acid (EDTA) and 2% insoluble polyvinylpyrrolidone in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulted supernatant was used for the determination of enzyme activities. The whole extraction procedure was carried out at 4°C. SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart and Bewley (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 according to the method of Patra et al. (1978). POD activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation (Nickel and

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Cunningham, 1969). APX activity was measured by the decrease in absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981).

Statistical analysis

All data presented here are the mean values of three independent experiments with three replicates. All results were analyzed statistically by two-way ANOVA with SAS 9.1.3 software and means were compared with the LSD test ($P < 0.05$).

Results

Biomass

Effect of different concentrations of Zn (0, 100, 300 and 500 µM) and interaction with sodium nitroprusside (100 and 200 µM) on plant growth, expressed as the total dry weight shown in *Table 1*. Zn exposure inhibited the growth of *Plantago major* significantly compared with control (Zn0Snp0), however, this inhibition was alleviated by the additions of 100 µM SNP. When applying 200 µM SNP into Zn-treated solution, Zn-induced inhibition on plant growth was not mitigated and the total dry weight reduced (*Table 1*).

Photosynthetic pigments (chlorophyll a and b)

A significant decrease in chlorophyll a and b contents was observed in the leaves of *Plantago major*, which were exposed to Zn stress. Compared with Zn-stressed plants, the addition of 100 µM SNP alleviated Zn toxicity in the photosynthetic apparatus. Whereas high concentration of SNP (200 µM) had no alleviated effects on the Zn-decreased chlorophyll contents (*Table 1*).

Hydrogen peroxide content

H_2O_2 content in leaf and roots of *Plantago major* was increased depending on Zn concentrations. Under SNP concentrations, H_2O_2 content was decreased in low concentration and increased in high concentration of SNP. However, interactions between Zn and SNP concentrations showed that under Zn concentrations, the additions of SNP 100µM inhibited Zn-induced H_2O_2 generation significantly (*Table 1*). This result indicates that the accumulation of H_2O_2 can reflect the oxidative stress and the changes of antioxidant in plants and indicates that low concentration of NO acts as an efficient ROS scavenger.

Malondialdehyde content

In the present study, the concentration of MDA was significantly increased ($P<0.05$) by Zn treatments in leaf and roots, which indicated an enhanced level of lipid peroxidation in metal-exposed plants. Under SNP concentrations, MDA content increases with increasing SNP concentrations. The application of SNP to Zn treatments shows that the low concentration of SNP (100 µM) can alleviate the effects of Zn stress on lipid peroxidation (*Table 1*).

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Table 1. *Effects of different concentrations of SNP on total dry weight, Chl a and b, Leaf and Root MDA content, Leaf and Root H_2O_2 content in Plantago major under Zn stress*

Zn	SNP	Total dry weight (gr)	Chl a (mg/g FW)	Chl b (mg/g FW)	Leaf MDA content (nmol. g^{-1}.FW)	Root MDA content (nmol. g^{-1}.FW)	Leaf H_2O_2 content (µmol. g^{-1}.FW)	Root H_2O_2 content (µmol. g^{-1}.FW)
0	0	0.743 a	2.767 ab	0.76067 a	26.5 j	17.563 j	22.737 i	17.46 g
	100	0.71 b	2.837 a	0.7623 a	27.417 i	17.993 j	23.073 i	17.267 g
	200	0.63 c	2.567 bcd	0.727 b	33.657 g	21.444 i	24.93 h	19.733 f
100	0	0.69 b	2.433 de	0.7273 b	34.727 f	25.163 h	26.956 g	22.123 e
	100	0.75 a	2.673 abc	0.7723 a	30.646 h	21.96 i	25.213 h	21.903 e
	200	0.633 c	2.313 e	0.7177 b	36.33 de	26.693 g	27.393 f	23.863 d
300	0	0.6 d	2.02 f	0.6206 d	36.123 e	32.327 e	28.507 e	23.766 d
	100	0.687 b	2.47 cde	0.687 c	33.35 g	28.587 f	26.83 g	21.833 e
	200	0.567 e	1.753 g	0.5943 e	37.016 d	34.373 c	29.703 d	24.977 c
500	0	0.47 f	1.45 h	0.462 g	40.203 b	37.7533 b	32.863 b	25.583 b
	100	0.55 e	2.02 f	0.5417 f	38.513 c	33.62 d	30.326 c	23.516 d
	200	0.43 g	1.24 i	0.4126 h	43.97 a	41.18 a	34.416 a	26.326 a

Means followed by the same letter are not significantly different (P < 0.05).

Peroxidase enzyme activity

According to *Figs. 1* and *2*, leaf and root POD enzyme activity increased significantly (at 0.05 level) under Zn stress condition. Under SNP concentration enzyme activity increased significantly (at 0.05 level) in 100 µM and decreased significantly (at 0.05 level) in 200 µM. Interaction between Zn and SNP shows that enzyme activity increased significantly in Zn+SNP100 µM and decreased significantly in Zn+SNP200 µM. It seems that NO in low concentration can improve negative effects of Zn stress (*Figs. 1* and *2*).

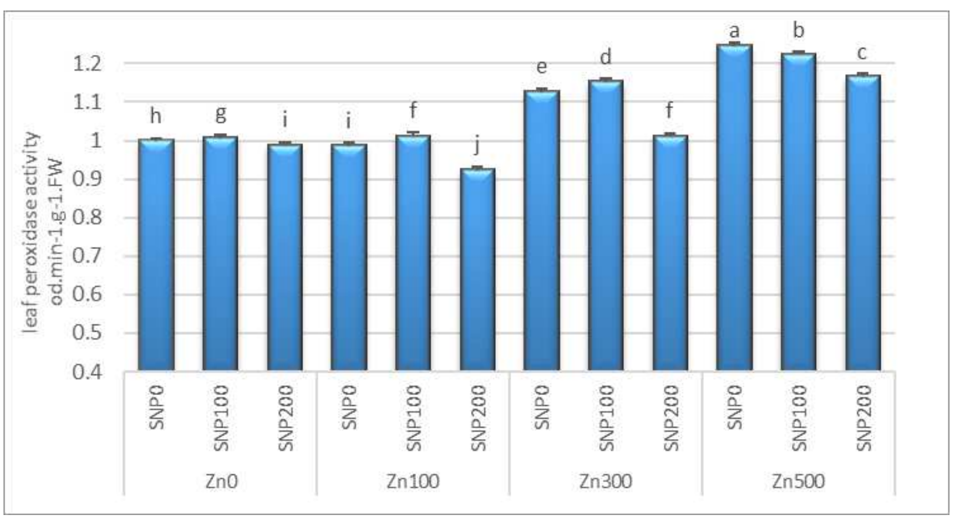

Figure 1. *Effects of different concentrations of SNP on peroxidase activity in leaf of Plantago major under Zn stress (P<0.05)*

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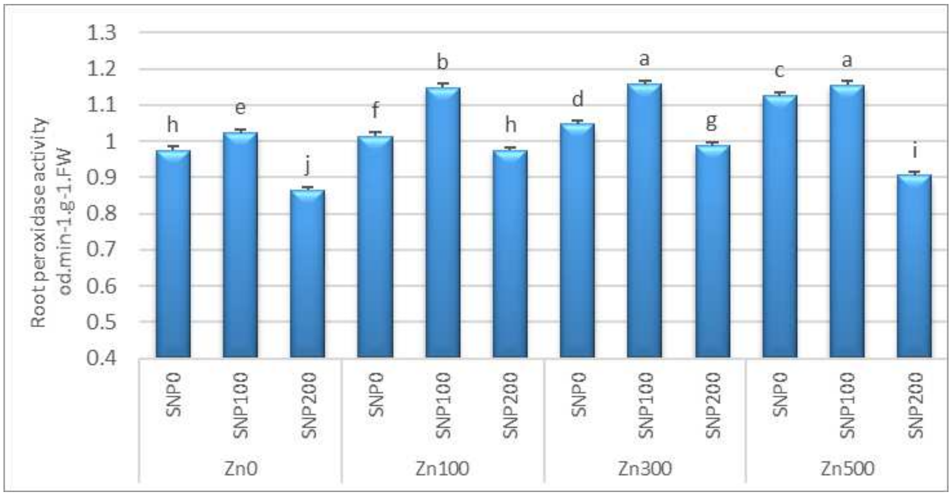

Figure 2. *Effects of different concentrations of SNP on peroxidase activity in root of Plantago major under Zn stress (P<0.05)*

Catalase enzyme activity

CAT enzyme activity increased significantly (at 0.05 level) under Zn stress condition compared with the control plant .Under SNP condition, there was decrease in SNP200 compared with SNP100. Generally, Zn treatment induced the activities of CAT in shoot and root of *plantago major* but this increase was found reversed in 200 µM SNP treatment (*Figs. 3* and *4*).

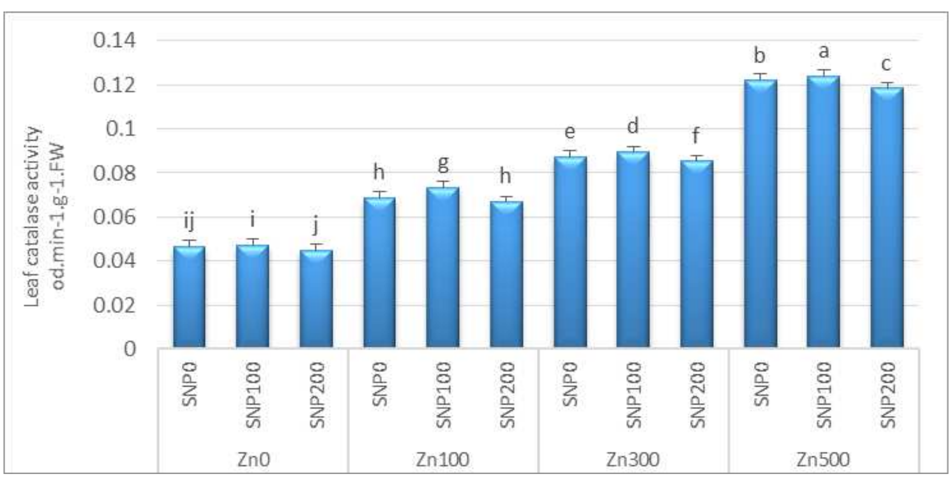

Figure 3. *Effects of different concentrations of SNP on catalase activity in leaf of Plantago major under Zn stress (P<0.05)*

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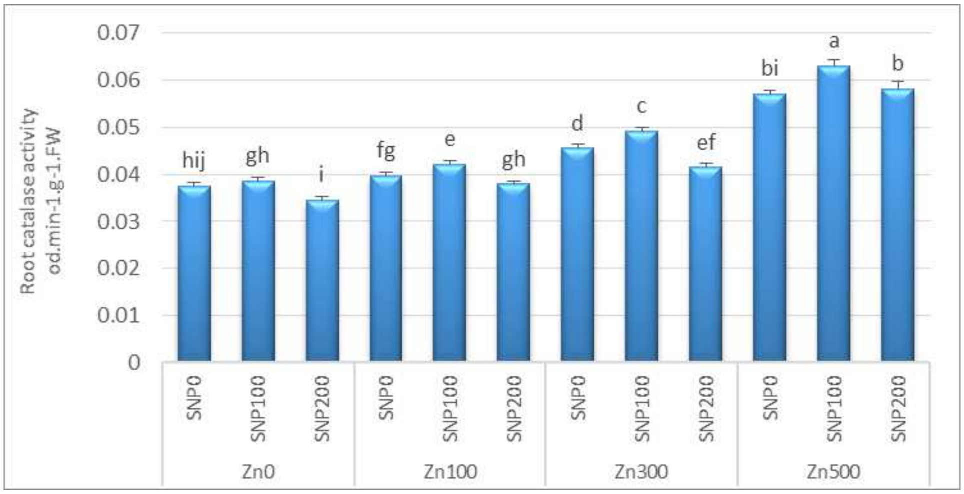

Figure 4. *Effects of different concentrations of SNP on catalase activity in root of Plantago major under Zn stress*

Ascorbate peroxidase enzyme activity

Leaf APX enzyme activity decreased significantly (at 0.05 level) with an increase of Zn concentrations. Under SNP concentration, the maximum activity of enzyme was found in Zn+SNP100 and the minimum activity was identified in Zn+SNP200 (*Fig. 5*). Root APX enzyme activity under Zn stress increased significantly (0.05 level) from Zn0 to Zn100 and decreased significantly (0.05 level) with elevated concentrations of Zn. In addition, under different concentrations of Zn treatments and SNP, showed that enzyme activity significantly decreased (0.05 level) in Zn+SNP200 and significantly increased (0.05 level) in Zn+SNP100 (*Fig. 6*).

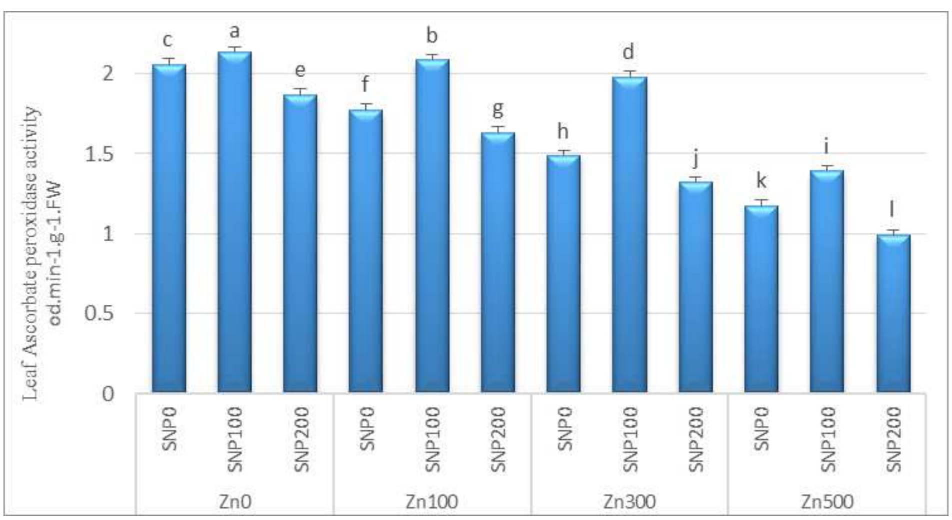

Figure 5. *Effects of different concentrations of SNP on ascorbate peroxidase activity in leaf of Plantago major under Zn stress*

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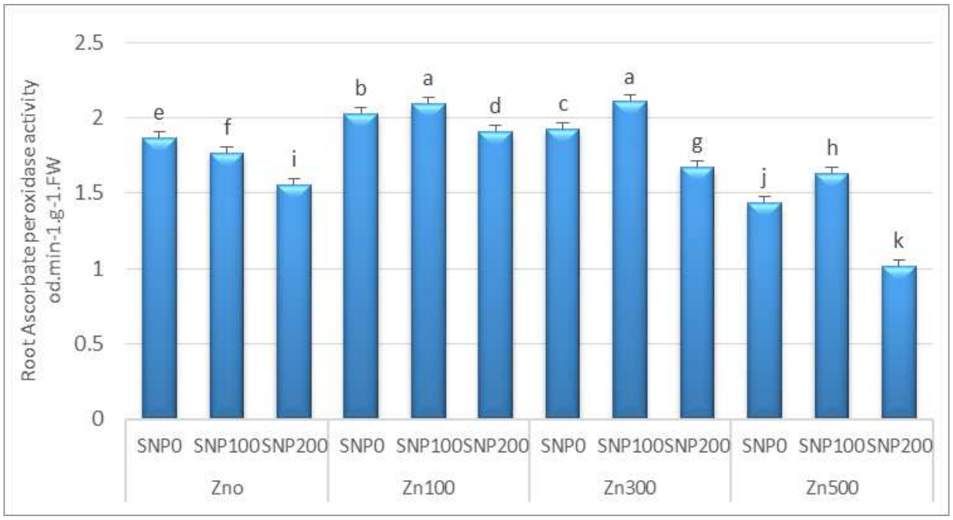

***Figure 6.** Effects of different concentrations of SNP on ascorbate peroxidase activity in root of Plantago major under Zn stress*

Superoxide dismutase enzyme activity

In leaf and root of *Plantago major*, the highest level of SOD activity was seen at 100 µM concentrations of Zn. Under SNP concentration, enzyme activity decreased significantly (0.05 level) by increasing SNP. Application of SNP to Zn concentrations shows that the highest level of activity was seen at Zn (0,100,300,500) +SNP100 and the lowest level of activity was seen at Zn (0,100,300,500) +SNP200. Hence, SOD activity was decreased depending on Zn concentrations, but the application of SNP200 µM caused a significant increase in SOD activity (*Figs. 7* and *8*).

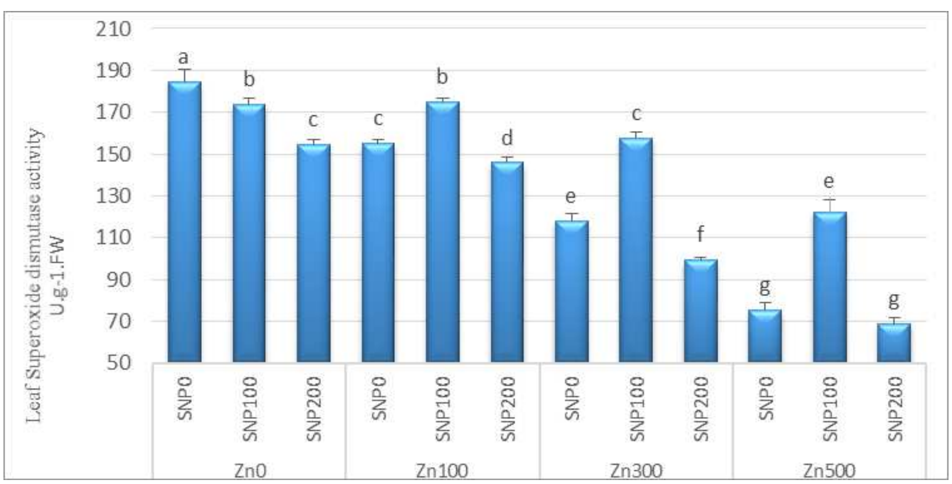

***Figure 7.** Effects of different concentrations of SNP on superoxide dismutase activity in leaf of Plantago major under Zn stress*

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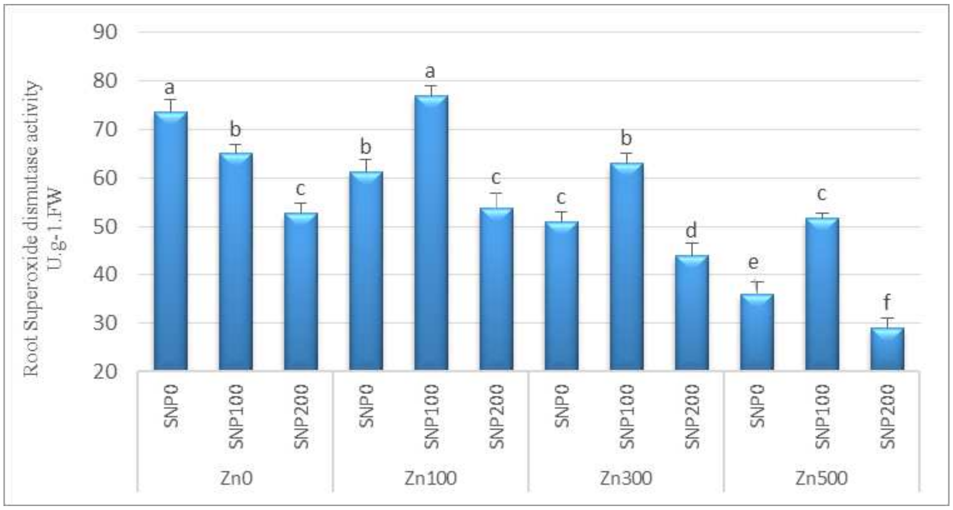

Figure 8. *Effects of different concentrations of SNP on superoxide dismutase activity in root of Plantago major under Zn stress*

Discussion

In our research, different NO concentrations were applied in Zn-treated growth medium. We investigated the physiological specifications of *Plantago* under Zn stress by using different NO concentration, aiming at finding a suitable concentration of NO to alleviate Zn toxicity effectively. The results of the present study indicated that application of Zn (100, 300 and, 500 µM) especially concentration of 500 µM decreased the dry weight of *Plantago*. However, simultaneous application of low concentration of NO (100 µM) increased the dry weight. Zn-induced growth inhibition was reflected by total dry weight. Inhibition of growth in *Plantago* plants might be the result of Zn caused variation of metabolic processes, such as oxidative damage, nutrient uptake and photosynthesis. Sheldon and Menzies (2005) reported similar results on Rhodes grass. However, Zn-induced inhibition was significantly reduced by the lower concentration of No. The mitigation effect of lower concentration of NO might be that NO induced the improvement of in photosynthesis by increasing chlorophyll content, counteracted oxidative damage by decreasing the generation of ROS. When the higher NO concentration was applied in *Plantago* plants, the mitigation effect was not obvious.

In this study, chlorophyll content showed maximum at 100 µM of Zn and it was decreased beyond that concentration. The decrease in chlorophyll content is believed to be because of: (1) inhibition of enzymes associated with chlorophyll biosynthesis (John et al., 2009); (2) inhibition of uptake and transportation of other metal elements such as Mn and Fe by adversary effects (Jayakumar et al., 2009; John et al., 2009). These results are in agreement with Yuanjie et al. (2013) who reported that Cu significantly decrease total chlorophyll, chl. a and b as compared with the control. The applications of different concentrations of SNP changed the chlorophyll contents in ryegrass. In another research, a similar reduction in total chlorophyll content under heavy metal toxicity was observed in *Atriplex halimus* (Brahim

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and Mohamed, 2011) and tobacco plants (Kheiry et al., 2016). Similar decrease in chlorophyll content under Cu stress was reported in *Atriplex halimus*. Our results shows that the application of exogenous NO at lower concentration (100 µM) alleviated Zn-induced decrease in chlorophyll content. Our results indicated that NO mediated improvement of chlorophyll contents played a role in the enhancement of photosynthesis.

As an active redox metal, Zn is able to induce the overproduction of ROS, such as hydrogen peroxide (H_2O_2), which in turn lead to lipid peroxidation and oxidative stress (Zhang et al., 2009). Results of this study showed that high concentrations of Zn, especially in 500 µM induced over accumulation of H_2O_2 and MDA in leaves and roots, leading to the oxidative damage in *Plantago* plants. However, application low concentration of NO (100 µM) alleviated H_2O_2 under Zn stress, protected cell membrane from peroxiding and decreased the accumulation of MDA. Similar to our results an increase in MDA and H_2O_2 content under excess nickel has been reported in *Eleusin*e *coracana* L. (Viswanath et al., 2016).

The levels of lipid peroxidation in the plant cells are measured by the determination of their MDA content, a breakdown product of lipid peroxidation. A high level of MDA is expressive of an enhanced formation of ROS and oxidative damage. In fact, ROS delete hydrogen from unsaturated fatty acids and generates lipid radicals and reactive aldehydes, which distort the lipid bilayer (Mishra et al., 2006). The results in the present work are in adaptation with the explanation of Panda et al. (2003), who reported heavy metal induced oxidative stress in Wheat leaves cell. Zhang et al. (2014) investigated that excess Zn altered the redox status of the aquatic plants (*Hydrilla verticillata*). In support, Chao et al. (2008) reported rapid generation of H_2O_2 by Zn-treated plant cells.

To cope with ROS and alleviate their toxic effects, plants have different antioxidation mechanisms (mediated by both enzymatic and non enzymatic antioxidants) operational in them to take care of the deleterious ROS (Puthur, 2016). Antioxidant system plays an important role in plant tolerance to stress conditions, which is based on the fact that the activity of one or more of these enzymes or antioxidant substances in general increase in plants when exposed to stressful condition and these enhances are related to increased stress tolerance (Fecht-Christoffers et al., 2003).

In our experiment, results shows that activities of antioxidant enzymes such as SOD and APX were decreased with increasing Zn concentrations but POD and CAT were increased. However, supply of exogenous NO, mainly 100 µM improved the Zn-alteration on antioxidant enzyme activity, which played a role in alleviating Zn-induced oxidative damage. Many studies have found a decrease (Malar et al., 2014) or increase (Xu et al., 2014) in activity of antioxidant enzymes under metal stress.

Previous reports revealed a variable response of an increase or decrease in SOD activity in plants exposed to different metals including Zn (Dixit et al., 2001). SOD is an essential component of antioxidant defense system in plants and it dismutase two superoxide radicals to water and molecular oxygen (Magdy and Azooz, 2013). A reduction in SOD activity in the metal treated plants has been attributed to an inactivation of the enzyme by H_2O_2 that is formed in different cellular compartments where SOD catalyzes the scavenging of superoxide radicals. SOD activity has been reported to increase under Zn toxicity (Kupper et al., 2007). Increase in SOD activity in response to stress appears to be probably due to de-novo synthesis of the enzymes protein (Erdei et al., 2002). Magdy and Azooz (2013) indicated that Zn treatment induced the activities of CAT and APX in shoots of *Hibiscus*

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esculentus. CAT is universally present oxidoreductase that decomposes H_20_2 to water and molecular oxygen and it is one of the key enzymes involved in removal of toxic peroxides. CAT along with APX and SOD are considered as vital enzymes within the antioxidant defense mechanism, which directly determine the cellular concentration of H_2O_2 (El-Shora, 2004). All the antioxidant enzymes studied in this work explain maximum activity in leaf compared to roots. It might be due to translocation of Zn in aerial parts as a micronutrient and this augmented the concentration of antioxidant enzymes in leaf compared to roots. The increased antioxidant enzyme activity might be ascribed to the role of NO in stimulating the expression of their genes (Xiong et al., 2010; Shi et al., 2016). Two mechanisms, which may explain NO protective action against oxidative damage, have been reported. Firstly, NO might detoxify ROS directly, such as superoxide anion, to form peroxyntrite, which is less toxic and thus limit cellular damage (Martinez et al., 2000). Secondly, NO could function as a signaling molecule, which activates the cellular antioxidant system (Lamattina et al., 2003). In the present study, the application of high NO concentration did not reduce Zn-induced ROS damage, even produced more toxic effects in *Plantago major.* However, the effects of NO depend on its concentration. Lamattina et al. (2003) reported that NO might regulate the expression of antioxidative genes to stimulate the relative enzyme activities. NO-mediated increase in one or more of these antioxidant enzymes contributed to the enhancement of antioxidative ability. Such results suggested that the protective effects of NO on oxidative damage were partly related to its role in up-regulating antioxidative ability. Universally, NO, as a recognized regulator of protein activation by S-nitrosylation, might inactivate heavy metal by modification of phytochelatins or some other ligands containing SH-group (Fecht-Christoffers et al., 2003). Finally NO can function as a signaling molecule for the cascade of events that lead to changes in gene expression under risk element stress (Procházková et al., 2012).

In conclusion ,our results demonstrated that in the hydroponics experiment, the alleviation effects of NO on Zn stress in *Plantago* needed a suitable concentration and the lower concentration of NO (100 μM) had a higher protective effect on Zn toxicity, while high concentration of NO (200 μM) did not alleviate Zn toxicity effectively. Our study proved that exogenous NO at low concentrations increased Zn tolerance in *Plantago* grown in Zn–mediated nutrient solution by (1) improving antioxidant enzyme activities and protecting against Zn–induced oxidative stress, (2) reversed the Zn toxicity effect and decreased the MDA content and H_2O_2 accumulation. This indicates that NO acts as an efficient ROS scavenger and membrane stabilizer in *Plantago* plants exposed to Zn stress.

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RAPID ENVIRONMENTAL CHANGES IN THE WESTERN ANTARCTIC PENINSULA REGION DUE TO CLIMATE CHANGE AND HUMAN ACTIVITY

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Abstract. The Antarctic and the Southern Ocean are a critically important part of the Earth system. The climatic, physical, and biological properties of this region are closely linked to other parts of the global environment. 200 years of direct human impact, recent climate amelioration and changes in the main sources and circulation of biogenic compounds as well as accumulation of industrial contaminants have significantly affected the whole ecosystem. Particularly sensitive is the region of the Western Antarctic Peninsula, which is considered to be one of the hot spots of the Earth. In this paper, we review recent literature and compare it with historical data to estimate and predict the consequences of this process. The Antarctic ecosystems can no longer be regarded as pristine. Global as well as local human influence has transgressed the barriers isolating that continent from the rest of the World, causing previously observed changes to accelerate.

Introduction

The specificity of Antarctic conditions

Antarctica is surrounded by the Southern Ocean. Together they occupy about one-third of the southern hemisphere. The Antarctic continent and associated islands are situated at a distance of nearly 1000 km from South America and of 4000–5000 km from Australia and South Africa. The isolation process began with the breakup of Gondwana and was enhanced and then maintained by the development of the atmospheric Polar Vortex and oceanic Antarctic Polar Front (Clarke et al., 2005; Barnes et al., 2006). Terrestrial ecosystem development is limited to only 0.34% of ice-free areas of the whole surface of Antarctica, mainly concentrated on coastal zones of the

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Antarctic Peninsula (Convey et al., 2009). This region is characterized by insular occurrence of exposed ice-free grounds and also by patchy and discontinuous soil distribution, displaying one of the harshest environmental conditions found on Earth (Convey et al., 2014). Low temperatures, limited liquid water availability, a specific light regime, elevated ultraviolet-B radiation levels, desiccating and destructively strong winds, poorly developed soils with low organic matter and nutrient content, slow organic matter decomposition, irregular nutrient distribution (from nutrient-deficient habitats to the ones extremely enriched in nutrients by e.g. huge breeding colonies of seabirds), high salinity in many locations (due to marine influences), the presence of permafrost, freeze–thaw events, cryoturbation, solifluction or ablation significantly limit the development of terrestrial communities (Chwedorzewska, 2009; Convey et al., 2014). Thus, characteristic features of Antarctic organisms are high stress tolerance, wide ecological amplitude but with minor competitive ability or investment in dispersal strategies, reduced reproductive investment and output, and extended lifespans and life cycles (Convey, 1996, 2000). Variability in abiotic conditions is recognized as a key feature affecting organism responses (e.g. Tufto, 2000). Antarctic terrestrial organisms display biochemical, physiological and anatomical adaptations that allow them to withstand prolonged periods of freezing, desiccation and abrasion (Olech, 2004; Giełwanowska and Szczuka, 2005; Wasley et al., 2006; Ochyra et al., 2008).

Most taxa are represented in the Southern Ocean, with Antarctica's shelf waters are particularly rich in this regard (Brey et al., 1994; Clarke and Johnston, 2003; Siciński et al., 2011). Moreover, biomass and productivity of both oceanic and near shore systems are massive. The Antarctic marine environment is certainly characterized by extreme physical characteristics, most notably low water and air temperatures, annual extremes in light regime, wind speeds, disturbance and isolation. Yet, the Southern Ocean is the most thermally stable environment on Earth. Sea temperature fluctuation in Antarctica is seasonal, but unlike other environments, temperatures are remarkably stable in winter. Salinity in the Southern Ocean is also highly constant, even in near shore waters. The constraints for marine life are characterized by resource limitation associated with strong seasonality (Peck et al., 2005). Phytoplankton growth is restricted to a maximum of 3–4 summer months. Thus, the phytoplankton-dependent organisms endure a winter of low food supply. Unlike terrestrial species, marine organisms do not freeze and must, therefore, maintain physiologically active tissues throughout the year, utilizing strategies that minimize losses in winter (Peck et al., 2006).

Literature review

Climate change in the Antarctic

Climate change in Antarctica has been characterized by regional differences. So far the Western Antarctic Peninsula – the repository of most Antarctic terrestrial biodiversity is warming rapidly (Nicolas and Bromwich, 2014), partly due to the southern shift in the polar jet stream (fast flowing, narrow, meandering air current located near the altitude of the tropopause and westerly winds; Robinson and Erickson, 2014).

Temperature and water availability are the most important factors for the biology of Antarctic terrestrial organisms. Thus, even a small shift, especially in precipitation and liquid water availability, may have a profound biological impact on sensitive polar ecosystems (Chwedorzewska, 2009). Especially the maritime Antarctic region experiences the most pronounced and rapid changes regarding those factors. The

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temperature records collected in the Antarctic Peninsula region during the last fifty years show its warming by about 0.5°C per decade (Bromwich et al., 2013). This recent rapid regional temperature increase has caused glacier retreat (Smith et al., 1999), snow cover reduction (Fox and Cooper, 1998), an increase in the duration of the warm period and in the number of cumulative days with temperature above 0°C (Vaughan, 2006). Earlier spring thaws and later autumn freeze may have already significantly extended the vegetation season. Climate warming is also associated with an increase in availability of liquid water, which is more important for terrestrial communities than the increase in temperature alone. There were also changes in cyclonic activity patterns around the Antarctic, followed by changes in precipitation intensity and form (precipitation increasingly occurs as rain) (Bromwich et al., 2013; Kennedy, 1993; Quayle, 2003) with noticeable increase in total precipitation during the year (Turner et al., 2005). Beside direct precipitation, water availability in terrestrial habitats is governed by seasonal snow and glacial melt. In a range of maritime Antarctic sites, rapid rates of glacial melting and loss of 'permanent' snow banks have been observed (e.g. Fox and Cooper, 1998; Quayle, 2003). The increase in temperature cause rapid and earlier snow and ice cover melting, thus may exhaust freshwater reserves before the end of the vegetation season, increasing the risk of local drought. Furthermore, as the warming increases the frequency of winter thaws, it may lead to sub-snow ice layer formation on the ground surface, which has a destructive impact on terrestrial organisms (Arnold et al., 2003). Recently, a rapid glacier retreat in the whole Antarctic Peninsula region has been observed, causing emergence of vast postglacial areas (Favier et al., 2014). Permafrost is a very characteristic feature of Polar Regions. It restricts the water exchange to the active layer that thaws on a seasonal basis. Vegetation, the active layer, and the underlying permafrost are strongly linked, being key components of terrestrial ecosystems. Permafrost warming and active layer thickening were mainly attributed to mean air temperature rise, although in several cases the role of snow cover, soil properties and the overlying vegetation was emphasized (Romanovsky et al., 2010; Guglielmin et al., 2014). Over shorter timescales, the rise in temperature can have a strong effect on permafrost degradation. Withdrawal of permafrost due to warming increasingly contributes to nutrients loads in the subsurface waters. Soil frozen for thousands of years releases both water and nutrients, therefore seasonally the melt water can be extremely rich in biogenic elements, mainly phosphorus (Hobbie et al., 1999). Increased nutrient inflow has the potential to alter trophic interactions in Antarctic terrestrial ecosystems (e.g. Laybourn-Parry, 2003; Nędzarek et al., 2014). Deglaciation, intensification of weathering processes and permafrost decline, along with changes in the abundance of breeding populations of birds and pinnipeds (which have direct impact on the amount of nutrients transferred onto the land from the sea) cause dynamic changes in nutrient distribution and availability (e.g. Clarke et al., 2007; Smale and Barnes, 2008; Montes-Hugo et al., 2009). All those changes lead to a disturbance of terrestrial and freshwater communities, causing biodiversity decrease or/and changes in species composition. Huge amounts of meltwater coming from glaciers alter the freshwater balance in terrestrial ecosystems and at the same time generate an inflow of large amounts of nutrients to the sea. It also reduces the salinity of water in the coastal marine zone. Melting glaciers are causing a huge inflow of mineral suspension into the sea that results in silting of the bottom and affects the benthic communities as well as production of phytoplankton. At open sea, changes in the range of sea ice cover have been observed, which is also linked with the phytoplankton and zooplankton

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productivity (Clarke et al., 2007; Smale and Barnes, 2008). Furthermore, glacial meltwaters carry a substantial load of microbial cells. Castello and Rogers (2005) suggest that between 1 x 10^{17} and 1 x 10^{21} viable microorganisms are liberated each year by global glacier melt. Those microbes may have a profound influence on the composition of terrestrial and marine microbial communities, either by addition of highly competitive opportunists or providing genes of adaptive value (Świątecki et al., 2010; Dziewit et al., 2013; Zdanowski et al., 2013).

Global human impact

Antarctica is an area least affected by anthropogenic activity. However, recent regional rapid warming events of Western Antarctica have indicated that this area is as much affected by the impact of greenhouse gases like carbon dioxide, methane, nitrous oxide, ozone and chlorofluorocarbons as any other global site (Bargagli, 2008). Anthropogenic climate change not only directly affects the environment but also exerts other stressors.

The Southern Ocean circulation is a physicochemical boundary that isolates Antarctica from other oceans, therefore volatile contaminants reach Antarctica mainly via atmospheric transport from lower latitudes (Wania and Mackay, 1993; Wania, 2003; Choi et al., 2008). In the continental Antarctic, dense and cold air masses flow (katabatic winds) towards the coast. The katabatic drainage flow is compensated by the high troposphere air flow from the mid-latitudes, which also transports pollutants. This long-distance transport is affected both by circumpolar low-pressure systems and by the high pressure system over the Antarctic plateau (Shaw, 1998). This way, many Persistent Organic Pollutants (POPs) such as hexachlorobenzene, hexachlorocyclohexanes, aldrin, dieldrin, chlordane, endrin and heptachlor entered Antarctica and the Southern Ocean (e.g. www.unep.org). Snow precipitation is a very efficient mechanism for cleaning the air of airborne contaminants since snow can collect those compounds, transport them to the ground and accumulate in successive layers. Global distillation or fractionation by condensation in cold environments have been proposed as mechanisms whereby the Polar Regions may become sinks for some POPs (Wania and Mackay, 1993). Degradation of deposited POPs is very slow in the Polar Regions due to low temperatures and winter darkness. Most of the POPs get entrapped and incorporated into the Antarctic ice cap (Bennett et al., 2015).

POP concentrations in Antarctic organisms were generally low as compared to those reported for marine and terrestrial species at lower latitudes, and they are still among the lowest in the world. Yet, POPs are recorded in every level of the trophic chain and the phenomenon of biomagnification plays a more important role than the bioaccumulation itself (Szopińska et al., 2016), due to specific physiology and ecology of Antarctic organisms, especially their longer life spans. Top predators have a high risk of becoming sinks for POPS. Indeed, in some species the levels reported were occasionally high and comparable to those found in regions with a strong human impact (Corsolini, 2009).

Direct human impacts

Antarctica never had indigenous human populations. However, over the last 200 years, exploitation, exploration and research along with recent regional climate changes have significantly affected this remote region (Chwedorzewska, 2009). Some aspects of previous human disturbance of the Antarctic marine ecosystems still have particularly important implications, like the nineteen to mid-twentieth century commercial

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exploitation of fur seals and whales, which led to almost total extinction of these species in the Southern Ocean (Fraser et al., 1992).

In the last three decades, sudden development of scientific and tourist activity have been noted (Montes-Hugo et al., 2009; Chwedorzewska and Korczak, 2010). Every year a considerable number of tourists visit Antarctica, particularly the Antarctic Peninsula region (*Figure 1*). Combined with a number of scientific expeditions accompanied by huge amounts of cargo and equipment, human activity exerts noticeable impact on terrestrial ecosystems (Chwedorzewska and Korczak, 2010; Hughes et al., 2012). This activity requires the use of generators and heavy vehicles as well as construction of buildings, landing strips, roads, fuel reservoirs, etc. Waste disposal and incineration, sewage management and fuel combustion are further points of concern. Supply delivery to the stations, which usually takes place at the beginning of the Antarctic summer, are logistically complicated operations which require the use of heavy marine and land machinery to transport up to several thousand tons of cargo (Rakusa-Suszczewski and Krzyszowska, 1991). These procedures can have a strong effect on surrounding ecosystems, locally generating chemical pollution and directly damaging or altering soil, vegetation and freshwater ecosystems (Hale et al., 2008).

The highest impact has been reported around large stations like McMurdo Station, Ross Island (US Antarctic research centre) (Mazzera et al., 1999; Negri et al., 2006), whereas, according to research which has continued since the 1990s, the impact of small sized stations is rather minor (Rakusa-Suszczewski and Krzyszowska, 1991; Bargagli et al., 1998). Human activity associated with scientific research bases as well as with tourism concentrates mainly on small coastal ice-free areas with favourable topography and good microclimate condition (Rakusa-Suszczewski and Krzyszowska, 1991; Terauds et al., 2012) with developed tundra communities, and animal gathering sites (mainly huge breeding areas of sea birds and pinnipeds) (Chwedorzewska and Korczak, 2010). This combination indicates sites of high ecological value and sensitivity, which drastically magnifies the risk of deleterious human impacts.

Figure 1. *Tourists visiting Antarctic Station on King George Island (South Shetland Islands, Western Antarctica) in the austral summer (Photo: A. Znoj)*

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Biological contamination

Human presence in the Antarctic also brings other threats, potentially even more dangerous for the ecosystem than POPs. Climate change along with increased human activity in this region create opportunities for some outside species to invade and colonize maritime Antarctic (Hughes and Worland, 2010; Hughes et al., 2012; Chwedorzewska et al., 2015). In spite of the significant number of tourists visiting the Antarctic Peninsula region (www.iaato.com), the main colonization events seem to be associated with the supply routes of polar stations rather than with tourism (Lee and Chown, 2009; Chwedorzewska and Korczak, 2010; Chown et al., 2012). Packing materials, vehicles, imported fresh foodstuffs, adhered soil, scientific equipment, building materials, clothing and footwear are considered as a potential vectors for alien species propagules (e.g. Lityńska-Zając et al., 2012; Augustyniuk-Kram et al., 2013; Chwedorzewska et al., 2013). On the other hand, due to amelioration of environmental condition of the Antarctic Peninsula region and changes in cyclonic activities (Kennedy, 1993), increased appearance of non-native birds species from lower latitudes was observed (e.g. Korczak-Abshire et al., 2011; Gryz et al., 2015) which can be responsible for the spread of alien propagules or/and pathogens threatening native species (Grimaldi et al., 2011).

Rapid changes in Antarctic terrestrial ecosystem

One of the most visible effects of climate change in the Antarctic Peninsula region is the fluctuation of sea ice coverage and dramatic glacier retreat (Bromwich et al., 2013). This process can lead to changes in ocean productivity, like alterations in plankton community composition and the most important changes in krill (*Euphausia superba*) recruitment and abundance (Ducklow et al., 2007). Krill, a key species of the whole Antarctic marine ecosystem, is also the biggest protein source in oceans and forms a critical trophic link between primary producers and upper-level consumers (fish, pinnipeds, cetaceans, and marine birds). Krill is also a very valuable commercial resource, intensively exploited from the early 70s (Nicol et al., 2012; Murphy et al., 2013). Stability of terrestrial communities is directly connected with constant supply of fertilizer from the sea - mainly droppings of breeding sea birds and pinnipeds (krill consumers). Population size fluctuations due to climate change (e.g. Korczak-Abshire et al., 2012; Korczak-Abshire et al., 2013) and fishery can directly influence terrestrial communities e.g. particularly the structure of ornithocoprophilous vegetation (Chwedorzewska and Korczak, 2010). Such fluctuation has been recently observed in Antarctic fur seals (*Arctocephalus gazella*), marked by a very rapid increase in population size to the levels that are greater than those existing before their exploitation in the nineteenth century. The presence of large numbers of fur seals on land has led to the rapid deterioration and changes in terrestrial tundra communities over large areas of ground accessible from the coast, where the majority of well-developed terrestrial ecosystems are found. It has also led to the rapid eutrophication of limnetic ecosystems accessible to the fur seals (Favero-Longo et al., 2011).

In the maritime Antarctic, development of terrestrial communities is controlled by extreme environmental conditions rather than biotic interactions. Those communities are expected to be very sensitive to changes in climate or consequential processes (Bargagli, 2005; Frenot et al., 2005). The consequences are thought to include increased diversity, biomass and trophic complexity, all of which enable development of a more

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complex ecosystem. Changes in the natural distribution ranges of native species or entire communities are also predicted. In the face of weakening natural barriers isolating Antarctica, it may be possible for single species or even whole communities to migrate from temperate zones to Polar Regions and shift the vegetation zones towards higher latitudes (Convey, 2006). Thus, most likely, further complications will arise from the complexity of species interactions. Some alien species transferred by humans may rapidly adapt to new conditions and engage in competition with native species (Molina-Montenegro et al., 2012). The most studied examples of such an event is the appearance and expansion of the annual meadow grass *Poa annua* (*Figure 2*) in the vicinity of the Antarctic stations situated along the Antarctic Peninsula (e.g. Wódkiewicz et al., 2013; 2014; Molina-Montenegro et al., 2014; Galera et al., 2015; 2016; Kellmann-Sopyła and Giełwanowska, 2015; Kellmann-Sopyła et al., 2015; Giełwanowska and Kellmann-Sopyła, 2015). While the contemporary Antarctic biota show the ability to survive abiotic environmental extremes, its competitive abilities are very poorly developed and even whole communities are vulnerable to increased competition of opportunistic invaders (Bargagli, 2005; Chwedorzewska and Bednarek, 2011; Hughes et al., 2012; Androsiuk et al., 2015).

Figure 2. *Poa annua in the vicinity of Polish Antarctic Station "Arctowski" on King George Island, South Shetland Islands, Western Antarctica (Photo: A. Znoj)*

Expansion of the distribution ranges of indigenous species within Antarctica is also possible, since two native Antarctic species have been already identified as having long-distance dispersal potential (Convey and Lewis Smith, 1993) and are the most studied examples of a biological response to the recent environmental warming in maritime Antarctic (Convey and Lewis Smith, 1993; Gerighausen et al., 2003). Some populations have already increased by two orders of magnitude in size during the last three decades, and there has been a change in the balance of reproductive strategy utilization towards

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successful sexual reproduction by increasing the probability of establishment of germinating seedlings (Convey, 1996; McGraw and Day, 1997). A rapid increase in the population size has also been observed amongst bryophytes and microbiota in maritime Antarctic (Convey and Lewis Smith, 1993; Green et al., 1999). The most unpredictable and difficult to monitor is the appearance of alien microorganisms, including pathogens able to cause an epidemic (Kerry et al., 1999). Pollution, increased connectivity, and global environmental change affecting pathogens and their vectors' migration at high latitudes are likely to drive future disease emergence in this region (Grimaldi et al., 2011).

The most dramatic effect of climate warming in maritime Antarctic is the rapid retreat of glaciers (Zdanowski et al., 2013). Glacier forefields experience one of the most important vegetation changes: colonization and primary succession of huge areas devoid of vegetation uncovered from under the ice (Olech et al., 2011). Ice cap reduction may also profoundly increase the amount of mobile POPs contaminants entrapped so far in glaciers (secondary source of pollutants). Glacial melt may carry those pollutants to nearby lakes or coastal marine zones, thereby spreading them and increasing their entry into trophic webs (Rakusa-Suszczewski and Krzyszowska, 1991; Corsolini and Focardi, 2000; Ainley et al., 2005) causing increased POP accumulation in long-lived Antarctic organisms (Walther et al., 2002).

Discussion

Entire Antarctica, governed internationally by the decisions of the Antarctic Treaty countries, has a status of a natural reserve. Members of the Antarctic Treaty, the Consultative Parties, have committed themselves to the comprehensive protection of the Antarctic environment. The Protocol on Environmental Protection to the Antarctic Treaty (also known as the Madrid Protocol or Environmental Protocol, http://www.ats.aq/e/ep.htm) entered into force in 1998. This document puts in place a framework for the protection of Antarctica's environmental, scientific, historic, wilderness and aesthetic values. However, the Antarctic Protected Area system (http://www.ats.aq/documents/recatt/Att004_e.pdf) is still immature and further implementation of the existing management tools may be required to protect the diverse range of vulnerabilities, qualities and spatial scales represented in different fields of science (e.g. Hughes et al., 2015, 2016).

Conducting research on such a complex and remote ecosystem needs critical comparison over a long period. The main problem is that scientific activity in the Antarctic is very recent, for example, the first very robust meteorological records came from the 1950s from Faraday Station and the first data on the occurrence of anthropogenic pollutants comes from the 1960s. There are a lot of gaps in data sequences. Due to high costs of expeditions and very harsh environmental conditions, vast areas are still unexplored, with some places inaccessible. The development of Antarctic research has been conducted in fact for only a few decades, over which analytical methods have undergone continuous changes, thus making comparison with the oldest data very hard and sometimes even impossible. Often, information is scarce or lacking very crucial data, for example data concerning the biology of the sampled species, lack of knowledge of baseline conditions or concurrent natural phenomena that mask their effects, also research results are presented in various units, which makes them difficult to compare (Szopińska et al., 2016).

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Most importantly, in many fields, there is no proper long-term monitoring system, or it is only now being developing. Rapid changes in the Antarctic ecosystem are becoming a serious issue that needs to be more effectively investigated. In fact, in Antarctica, only the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) manages sustainable fishery in this region, running very broad, long-term monitoring (CCAMLR Ecosystem Monitoring Program – CEMP), which started in the late eighties. CEMP's major function is to monitor the key life-history parameters of selected dependent species to detect changes in the abundance of those being harvested. ‘Dependent species’ are marine predators for which species targeted by commercial fisheries are a major component of their diet (https://www.ccamlr.org). Officially launched at the end of 2011 and still developing was the Southern Ocean Observation System (SOOS), which seems to fulfill the needs of a modern monitoring system. SOOS is an international initiative of the Scientific Committee on Antarctic Research (SCAR) and the Scientific Committee on Oceanic Research (SCOR). SOOS mission is to facilitate the collection and delivery of essential observations of dynamics and changes in the physical, chemical, geological and biological parameters of the Southern Ocean system to an all international scientific community to advance understanding of the Southern Ocean and to address critical societal challenges. The SOOS tries to develop a cyberinfrastructure, where marine assets would include a mixture of both autonomous and non-autonomous platforms. Combined with satellite remote sensing, the data would be relayed to ground stations in real time, where mathematical ocean models would produce near real-time state estimates of each of the parameters in the system (https://www.soos.aq).

Conclusions

Recent and historical selective overexploitation of some key species, unexpected increase in the population of some other, anthropogenic climate change, alien species pressure, local production and long distance transportation of pollutants, their accumulation in the ice cap and very slow degradation in polar conditions show that Antarctic ecosystems can no longer be regarded as pristine. Global anthropogenic activities along with local human influence weakened the barriers that isolate this continent from the rest of the world, causing an acceleration of the already observed changes. The main problem in such remote regions like Antarctica is that some consequences of human pressure may remain undetected because of the lack of monitoring systems, limited knowledge of baseline conditions or concurrent natural phenomena that mask their effects (Tin et al., 2014). Thus, it is very important to develop new effective monitoring systems. In Antarctica, the remote sensing techniques have opened up new opportunities for ecosystem monitoring. Satellites as well as manned and unmanned aircraft enable the collection of data from a local to global scale, which can be easily integrated with “ground” base data. The most promising seem to be very recent developments in the use of Unmanned Aerial Vehicles (UAVs) for remote sensing applications (e.g. Goetzendorf-Grabowski and Rodzewicz, 2016), which provide new opportunities for high-resolution ecosystem mapping and monitoring, but still, application of this technology to collect environmental data is a relatively new phenomenon in polar regions (e.g. Korczak-Abshire et al., 2016; Dąbski et al., 2017).

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POPULATION STRUCTURE OF A DOMINANT HALOXYLON SPECIES, ACROSS A HABITAT GRADIENT IN THE SOUTHERN GURBANTUNGGUT DESERT, CENTRAL ASIA

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Abstract. In the context of habitat change, widely distributed vegetation can serve as relevant barometers of ecosystems' sensitivity or resilience to disturbances. This study analyzed morphological variations in trees and recruits density, individual size and their spatial structure through Haloxylon population's field experiment in each sampling location. Our results showed a significant decrease in tree density from the south (4445 trees/ ha) to the central desert (481 trees/ ha), whereas basal diameter, crown radius and height showed different tendency. Tree basal diameter and height structure of populations had positively skewed asymmetric distributions with "reverse J" shape. Tree density in the valleys between dunes (6133 trees/ha) was greatest. Few recruits were found in the central desert, but their density increased from central to south of the Gurbantunggut desert ,perhaps owing to seed rain patterns and availability of soil moisture and nutrients. Trees had a spatially clustered distribution in all study plots. Haloxylon spp. can tolerate wide climatic and habitat fluctuations, however recruitment can be unpredictable in harsh desert conditions. Soil physicochemical properties were the main ecological factor influencing spatial patterns. With increasing altitude, soil moisture and nutrient contents decreased significantly. In different dune habitats, Haloxylon population had different characteristics.
Keywords: *latitudinal variation; cluster; recruitment; morphological traits; spatial structure*

Introduction

Plant species with wide distribution ranges are exposed to a great variety of climatic and habitat conditions. Individuals and populations react to wide amplitudes of environmental factors through varying responses (Swenson and Enquist, 2007; Aitken et al., 2008; Wu et al., 2013). Developing broad adaptation strategies is particularly a requirement for species that are distributed along a large latitudinal gradient including both dry and humid areas (Aitken, 2008; Bognounou et al., 2009). Severe environmental degradation (e.g., decreases in river flows, a drop in underground water levels, overdevelopment in agriculture, overgrazing of animals, and mean temperature increases) has often led to an expansion of the desert area in the Gurbantunggut, causing high mortality in some woody desert species and radically changing their population structures (Huang et al., 2009; Allan et al., 2012; Si, 2010). The observed dwindling of natural Haloxylon forested areas has raised serious concerns for managers, scholars and scientists. One documented long-term consequence of anthropic disturbances is a shift

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in affected species' ranges (Lykke et al., 1999). However, although drought frequency and severity are predicted to increase across continental interiors, the consequences of these changes for dominant plant species are largely unknown (Mueller et al., 2005).

In the Gurbantunggut desert, there is little documentation of the distributions of plant species, and how these ranges are shaped by environmental factors is poorly understood (Qian et al., 2007). Several ecological investigations have examined the population structures and dynamics of some useful tree species in many parts of Asia and Africa, at both local (Nacoulma et al., 2011; Ivanov et al., 2008; Wu et al., 2013) and national scales(Sambare et al., 2011; Fandohan et al., 2011). However, few of these have specifically addressed widely distributed species in any great detail. This is the case for Haloxylon, that has an exceptional ecological amplitude, and includes the main woody species in central Asian desert ecosystems and artificial afforestation systems (Huang et al., 2009). It is also designated the king of psammophytic plants, owing to its important role in sand fixation, wind control and water conservation in the desert (Zhu et al., 2004). However, recent anthropogenic pressures, such as the expansion of agriculture, threaten both planted, artificial forests and natural populations of Haloxylon in China. Previous studies have suggested that Haloxylon needs effective conservation attention in natural stands in order to guarantee its persistence and to avoid a shortage of its products (Liang and Zhao, 1982). One of the main reasons for poor regeneration in Haloxylon is the high mortality of recruits (Si, 2010), likely because of very low water availability in soils. Understanding population patterns and underlying intrinsic characteristics is necessary for the conservation and sustainable management of widespread species distributed across biomes. Further more, the study of plant population characteristics is helpful for theoretical understanding of species coexistence and population structures, both of which are largely dependent on spatial patterns (Kikkawa and Anderson, 1984; Dale, 1999). Such quantitative and spatial patterns may be derived from processes and forces operating on different scales, such as heterogeneity in soil conditions, intra- and inter-specific competition, seed dispersal and other environmental factors (Jeltsch et al., 1999; Saei et al., 2014). For efficient conservation management of Haloxylon, detailed, large-scale analyses of its population ecology are necessary. The aim of this study was to document Haloxylon populations spanning a latitudinal gradient, from the extreme south of Gurbantunggut desert to the central desert. Specifically, we aimed to assess the structural patterns of populations spanning the latitudinal gradient; examine tree morphological traits (height, diameter, number of stems) across phytogeographical zones and analyze the spatial distribution patterns of Haloxylon individuals in natural stands.

Materials and methods

Study area

The study was conducted over the 2013 growing season, with three sampling transects situated in the Gurbantunggut desert (44º11'-46º 20'N, 84 31'-90 00'E), which is a part of Junggar Basin (*Fig. 2*) and has an area of 48 000 km^2. This desert is located in the hinterland of the Eurasian continent, within the Xinjiang region in northwest China. Haloxylon species are dominant components of the vegetation in the Gurbantunggut, as in all sandy and clay deserts across Central Asia. The desert is composed mainly of longitudinal dunes, although checkerboard- and honeycomb-shaped dunes are also present (Wang, 2003); he dunes vary in mobility from completely

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movable dunes, to semi-stable and stable dunes. The longitudinal dunes range in length from several hundred meters to tens of kilometers, have a height of 10-50 m, and generally run in a north to south direction (with the western slope very long and shallow, and the eastern slope short and steep) (Wang, 2002). Gurbantunggut desert has sandy soil and a continental, arid temperate climate, which is characterized by two contrasting seasons (cold winters and hot dry summers), owing to its great distance from the ocean. Seasons vary dramatically, with winter (December-February), spring (March-May), summer (June-August) and autumn (September-November) climatic conditions affecting plant growth cycles differently (Nyongesah and Wang, 2013). From 2003 to 2013, the coldest month is January (-19.50~-22.11℃) and the hottest month is July (23.13~27.69℃). The average annual temperature is 6.86~7.73℃. Precipitation ranges from 97.2 to 180mm, with winter and spring precipitation accounting for about two-thirds of the annual total (Qian et al., 2011). Moreover, precipitation varies spatially across the Gurbantunggut desert hinterland and surrounding areas (*Fig. 1*). The annual potential evaporation is 2000-2800mm (Song et al., 2011). Wind speeds are greatest during late spring each year, with an average velocity of 11.17m/s, and winds come predominantly from the west-northwest, northwest and north. Natural vegetation is sparse and the water table can be extremely deep (8-300m), although this is variable. In the study area, the vegetation is dominated by desert species, particularly Chenopodiaceae species, and species richness is relatively high. Common dwarf shrub and pygmy tree species include *Aristida pennata, Ceratocarpus arenarius, Ceratoides ewersmanniana, Ephedra distachya, Haloxylon ammodendron, Haloxylon persicum* and so on (Qian et al., 2003).

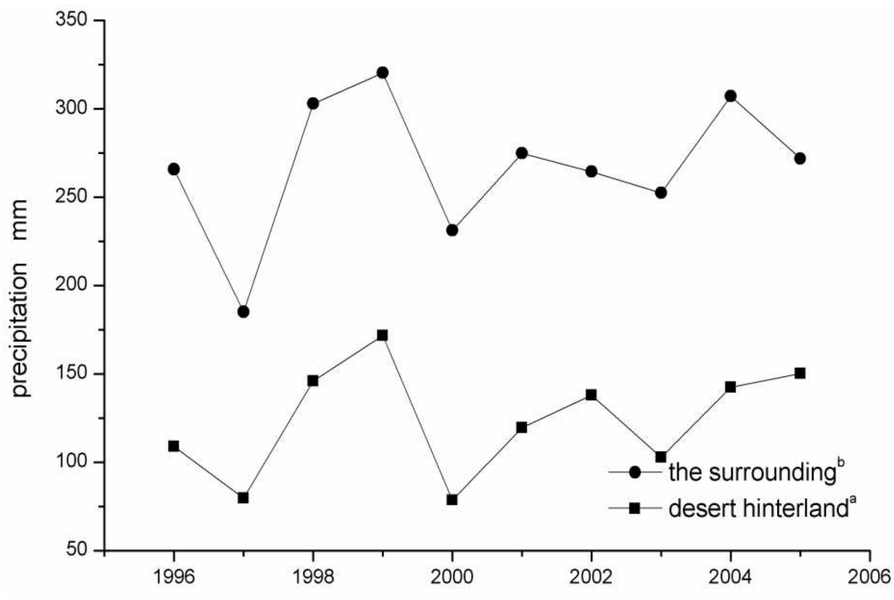

Figure 1. *Comparison of precipitation in the desert hinterland and surrounding lands*
[a]*Mean yearly precipitation was derived from the meteorological stations in the desert hinterland of shixi oil field (Liu et al., 2012).*
[b]*Mean yearly precipitation was derived from the meteorological stations of five surrounding cities (Karamay51243, Paotai51352, MoSuowan51353, Hutubi 51367, Fukang51377).*

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Sampling design

Combining recent field survey data with information on the distribution of dune types from previous reports (Liu and Zao, 2009), Google Earth was used to select three sampling transects among the longitudinal ridges from south to central Gurbantunggut Desert. The first transect was in a fixed dune-dominated area (F), the second a semi-fixed dune-dominated area (S) and the last a movable dune-dominated area (M); transects were 40km apart (*Fig. 2*). In each transect (1^{st} transect: 44º22'20.01N, 87º55'06.11E; 2^{nd} transect: 44º36'09.40 N, 88º14'17.83E; 3^{rd} transect: 44º56'52.01 N, 88º33'29.91E), four sampling locations were selected in accordance with the topographical characteristics of the dune: the valley between the dunes (valley), the east slope of the dune (leeward slope), the top (top) and the west slope (windward slope). Considering the dimensions of the sand dunes across transects, Haloxylon populations were surveyed in 10m×10m and 20m×20m main plots in each sampling location, with each main plot being associated with two sub-plots of 2 m×2 m to examine Haloxylon species regeneration patterns (individuals with dbh ≤ 0.3cm) and survey grass species. There were 20 main plots per transect which considered sufficient for reliable assessment of Haloxylon populations, according to previous prospecting of natural stands in the study area.

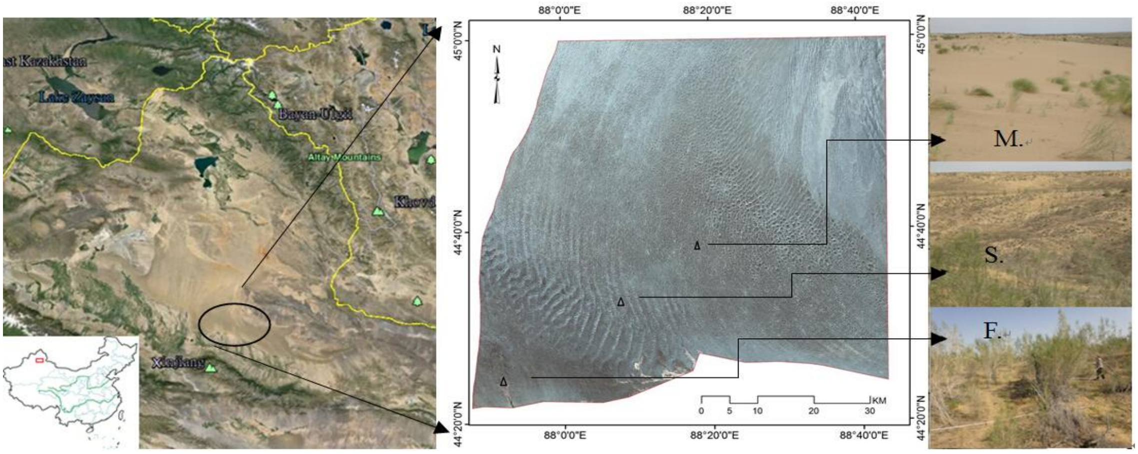

***Figure 2.** Position in the study area and sample design*

Habitat characteristics

The study area encompassed three different ecological (phytogeographical) zones (F, S, and M), covering different dune types (Liu and Zao, 2009), and an altitudinal gradient from north to central Gurbantunggut. The three different zones were comprised of different sizes of sand dunes with distinct vegetation coverage and sands of different particle sizes. Environmental data for each transect, including both climate and soil traits obtained from the nearest climate stations, are provided in *Table 1*. The extent of the snowy and rainy period varied slightly from north to central Gurbantunggut. As shown in *Table 1*, mean annual precipitation (data from 2000–2010) ranged from 110.4-128.6 (F) to 86-110 mm (M), and temperatures varied about 1-4°C (*Table 1*). The southern most edge of the fixed dune-dominated area (F) increased progressively in height towards the central desert, and hence soil available N, biomass, and organic carbon content, as well as conductivity, moisture and pH at 0-30cm soil depths

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decreased on average from the south (F) to the central (M) desert (*Fig. 3*). With the exception of electrical conductivity and effective N, soil parameters differed significantly among zones. According to survey data, there were more physical and biological crusts in the S versus F and M transect zones. Soil nutrient availability at the top of the dunes was significantly lower than in the other plot locations, whereas bottom plots (between the dunes) had slightly higher availabilities (*Fig. 3*). Soil moisture in bottom plots was significantly higher than in the other plot types (*Fig. 4*), and decreased from valley > east side> west side > top, which is consistent with previous reports (Li, 2013a). In the three transects, principal vegetation types included drought-tolerant plant species. The main human activities related to natural resource exploitation in the desert are agriculture and livestock farming.

Table 1. *Environmental factors describing the three different ecological/elevational study zones (fixed dune dominated desert area, F.; semi-fixed dune dominated desert area, S.; movable dune dominated desert area, M.)*

Parameter	F.	S.	M.
Mean precipitation	110–128.6[b]	100–123[c]	86–110[a]
Temperature (°C): min–max	-37–41.5	-35–39.7	-38.2–44.0
The average particle size of sand (Mz)	0.24	0.42	0.79
Elevation m	445.6	574.3	663.3
water content %	3.12	1.34	1.18
Water table (m)	8-12	30-50	>150
organic carbon g/kg	1.04	0.795	0.365
organic matter g/kg	1.79	1.37	0.63
Effective N g/kg	0.142	0.110	0.104
pH	8.00	7.83	7.69
Electrical conductivity ms/cm	0.114	0.089	0.064
physical and biological crusts	10-20%	30-60%	≤10%

[a,b]same as *Fig. 1*.
[c]Means yearly precipitation was derived from automatic monitoring performed in the desert management office.

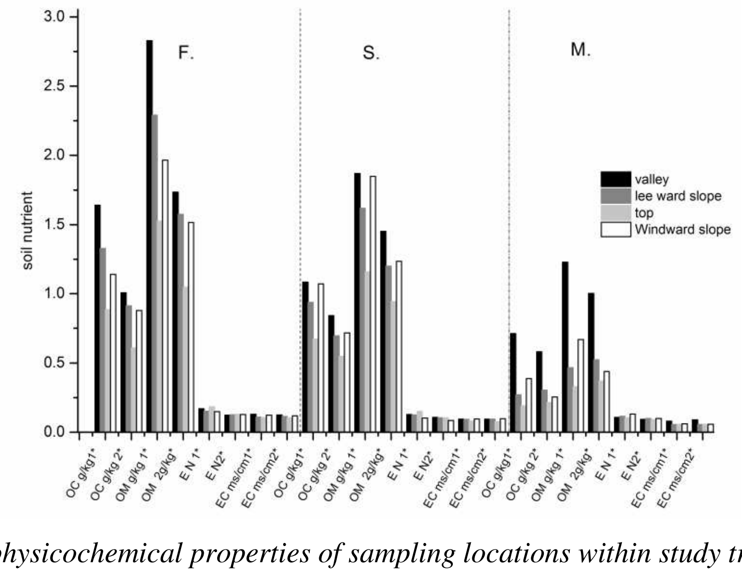

Figure 3. *Soil physicochemical properties of sampling locations within study transects (1*, 0-15 cm; 2*, 15-30cm; OC, organic carbon; OM, organic matter; EN, Effective nitrogen; EC, Electrical conductivity)*

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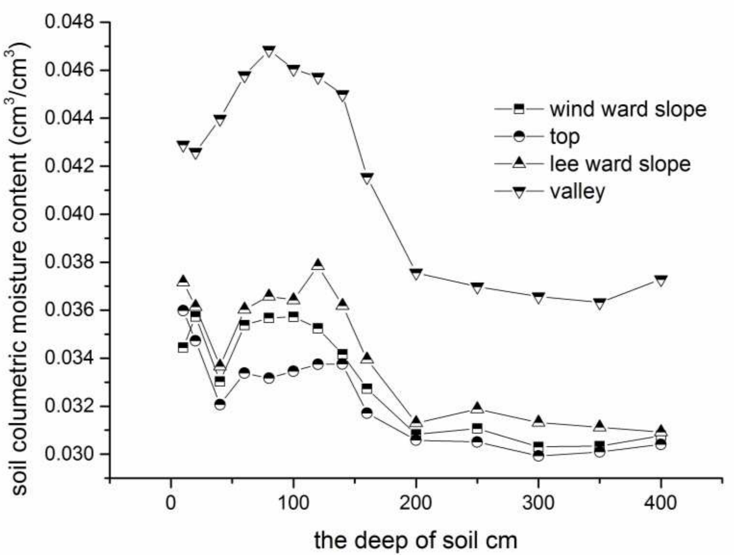

Figure 4. *Comparison of soil moisture at different sample locations in F*

Plant surveys and statistical analysis

In the study area, *Haloxylon ammodendron* and *Haloxylon persicum* are two dominant and keystone species. We pooled data for these two species as *Haloxylon persicum* occurred rarely and mainly on dune tops, and the two congeners are ecologically similar. Observations and measurements reported in this paper were made from May –July 2013. These dates roughly coincide with peak Haloxylon biomass at these localities (Hu, 1985). Haloxylon individuals were identified and measured in all plots for each transect; note that plots were arranged along an east to west sampling line. In each of the main plots, the following tree morphological parameters were measured: basal diameter, crown diameter, height and the number of stems (the number of branches off a main stem occurring below 0.5 m). The Cartesian coordinates (X-Y) of each tree were also recorded in the main plots. In the sub-plots, the height as well as the number of regenerating stems was determined for each individual. Habitat factors such as direction aspect, elevation, the geographical coordinates, slope and so on were also recorded. Soil samples were collected from random locations in the four corners and the middle of each plot at depths of 0-15 cm and 15-30cm. These were sealed in airtight containers plastic bags and taken for analysis in the lab. Soil moisture was measured using the neutron probe method and by oven-drying. After passing soil through a 0.25mm sieve, soil carbon and nitrogen contents were determined using the potassium chromate oxidation heating method-external heating method and the Kjeldahl method, respectively. The soil was washed with distilled water to measure pH and conductivity was obtained using the alternating current (AC) method.

In this study, undergrowth biomass was found by harvesting all aboveground herbaceous growth, drying to constant weight and then weighing the dried material (g/m^2). Shrub biomass was estimated non-destructively by building a testability-factor estimation model based on quantification theory (Zhao, 2004). The percent cover of herbaceous undergrowth was assessed visually in the 2m×2m sub-plots. The percent cover of shrubs was calculated using crown amplitudes and the sub-plot area. The number of individual plants was divided by the total area to get shrub density for each

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main plot in each type of sand dune. The average crown radius was calculated by averaging four measurements of shrub radii taken in different directions (N, E, S, W) (Gill et al., 2000).

Within a given environment, individuals of the same tree species and of the same age should respond to the environment in a consistent way (Frost and Rydin, 2000). Hence, basal diameter (the diameter of the main stem, i.e. ramet, or of the thickest and strongest stem if there was more than one) and height (the vertical height at the center of the plant) were used as indicators of individual size/age, in determining the composition of the population. In order to describe population structure in greater detail, the classification system used here for grouping plants differs from that of previous studies (Song et al., 2011). Plants were divided into several basal diameter and height classes. The number of individuals falling into each class was tallied for each transect to form a basic data set for population structure analysis. Histograms were used to help illustrate and aid in the interpretation of the diameter and height distributions.

Population dispersion indices are important indicators of the spatial distribution patterns of populations and may be calculated based on field survey data. Various indices have been proposed to describe the degree of spatial clustering in study populations, with different merits. In this study, using plot survey data, the DPS software package was used to calculate several aggregation indices for Haloxylon populations across transects and sampling locations.

Results and analysis

Primary population characteristics

Characteristics of Haloxylon populations found in all four dune habitats and three transects are summarized in *Table 2*, and reveal that average basal diameter, biomass, height and percent cover differed among transects and according to position on the dune. The biomass and percent cover of both Haloxylon and the grasses, as well as Haloxylon stand density, were significantly higher in F plots than in M or S plots. Plant biomass on the east side of the dunes was lowest in F, between dunes it was lowest in M, and on the dune tops lowest in S. *Table 2* reveals a significant decrease in Haloxylon population density from the south (4445 trees/ha) to the central desert (481 trees/ha), although the accompanying shrub species Calligonum leucocladum covered more area in the S versus M plots. Tree density between dunes (6133 trees/ha) was higher than in any of the other three sample locations on the dunes in F, but not in the other two zones. Trends in biomass and percent cover for Haloxylon and the grasses were consistent.In contrast to density, the tree morphological parameters (crown radius, diameter and height) showed a different trend across zones, increasing from F to S and then decreasing in M (*Table 2*). Among dune habitats, within the F area, dune tops had the largest average basal diameter at 4.11cm, whereas dune valleys had the lowest average basal diameter at 1.68 cm, owing to a high proportion of young seedlings. The mean basal diameter and height of Haloxylon individuals was greatest on dune tops in S. For the M zone, trees on the east-facing slope had the largest mean basal diameter and height. Apart from the size of the trees, the number of stems differed slightly among transects (*Table 2*), and the leaf area index decreased substantially from the south to central desert.

Looking at Haloxylon recruitment patterns, there were significant differences among populations from the different zones. There were few recruits in M or S, while the

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density of recruits was significantly higher in F, especially between dunes. Recruit density increased from central (52 individuals/ ha) to south Gurbantunggut desert (667 individuals/ ha). The relatively high recruit density in F may indicate a relatively younger population in F compared to M, perhaps due to more favorable habitat. Indeed, in F, recruits mainly occurred around established plants, where high soil moisture conditions may favor renewal; however, recruit survival and growth rates are extraordinarily low owing to high intraspecific competition (Wei, 2006). In the calculation of percent cover, physical and biological crusts were excluded. Haloxylon individuals of diameter between 0- 0.5cm were considered to be recruits.

Table 2. *Basic characteristics of Haloxylon populations in different habitats*

Terrain	**F.**					**S.**				
Characteristic parameters	valley	Lee ward slope	top	Wind ward slope	mean	valley	Lee ward slope	top	Wind ward slope	mean
biomass of Haloxylon (g. m^{-2})	710	652	673	685	680	120	140	350	260	217
biomass of grass (g. m^{-2})	54.4	38.6	44.5	43.96	45.3	37.6	53.9	41.5	53.46	46.6
Stand density	6133	3175	3280	3733	4445	325	561	635	545	516
Calligonum leucocladum density (strain /hm^2)	0	0	0	0	0	140	135	250	255	195
Coverage (%)	51.7	43.5	51.0	47.9	49	24.6	32.9	28.4	36.9	30.1
diameter (mean) (cm)	1.68	3.09	4.11	3.43	3.08	2.25	2.08	3.49	2.28	2.53
Height(mean) (cm)	48.7	147.2	136.8	144.5	119.3	110	81.7	123	81.47	99.0
crown radius (cm)	32.4	61.9	58.3	63.4	47.6	56.5	47.3	70.8	63.9	64.7
recruits density(strain /hm^2)	1300	20	0	140	365	0	180	140	0	80
The number of stems (ramification below 0.5 m)	1-8					1-18				

terrain	M.					total
Characteristic parameters	b valley	Lee ward slope	top	Wind ward slope	mean	dune
biomass of Haloxylon (g. m^{-2})	58	220	176.3	178	158	1391
biomass of grass (g. m^{-2})	45.73	30.7	10.7	24.25	27.6	40.8
Stand density	393	201	608	305	481	1701
Calligonum leucocladum density (strain /hm^2)	350	310	70	420	287	
Coverage (%)	14.8	13.9	7.81	9.27	11.0	30.5
diameter (mean) (cm)	2.75	3.54	2.09	2.17	2.64	2.75
Height(mean) (cm)	106.9	157.5	96.47	90.5	112.8	110.4

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crown radius (cm)	37.2	60.4	49.8	46.0	48.9	
recruits density(strain /hm^2)	0	60	60	140	65	170
The number of stems (ramification below 0.5 m)	1-22					
In the calculation of percent cover, physical and biological crusts were excluded. Haloxylon individuals of diameter between 0- 0.5cm were considered to be recruits.						

The basal diameter structure of Haloxylon populations in different habitats

Although grouping individuals based on stem diameter versus age may produce different distributions, the two methods are consistent in predicting species' responses to the environment (Frost and Rydin, 2000). Trees are often classified according to basal diameter or height, in order to analyze the population's structure (Liu et al., 2012; Song et al., 2008). Thus, in this study, diameter classes were used to gain insight into Haloxylon population characteristics. The basal diameter data for Haloxylon populations from different areas of the dunes, across the three transect zones, are presented in *Figure 5*. The basal diameter distributions were positively skewed (asymmetric) in all zones, with a "reverse J" shape. This indicates a predominance of small individuals (diameter of 0-3 cm), a pattern that was particularly marked in F (*Fig. 5*). The occurrence of young trees across transects reveals that Haloxylon populations are relatively stable with good regeneration potential. No individuals in the largest diameter class (more than 20 cm) were found on the east side of the dunes in F or in dune valleys in S and M. The average diameter of all trees surveyed in F (+4,000 trees) was 3.08cm, and more than 70 % of trees had a basal diameter of 3 cm or less, with trees of diameter 0-1cm accounting for the highest percentage of the total at 43%. There were only four plants with a basal diameter greater than 25 cm in the whole 2.4 hm^2 study area; these were located on dune tops in F and S zones, and on windward slopes in S and M zones. The largest basal diameter, 37cm, was recorded on the top of an F dune, however diameters between dunes in F were significantly lower than in other transects and sample locations. In the F transect, trees of small basal diameter (0-1cm) accounted for the greatest proportion (64%) of individuals on the plain between dunes, whereas in the other three dune habitats 1-2cm Haloxylon trees were the most common. The first peak of the fixed dune diameter distribution (dune valley populations) was the highest, but there was also a second peak in the next diameter class (other sample locations), which was different from the other zones. The distributions for dune top and leeward slope populations in S were relatively flat, with abundance gradually decreasing as basal diameter increased, but dune bottom and west slope populations peaked at 1-2cm. Dune top populations had a significantly higher proportion of trees of 10-20cm than other populations. Haloxylon trees of 6-7cm accounted for a significant proportion of the total in M plots, although the overall trend again showed that young recruits were predominant.

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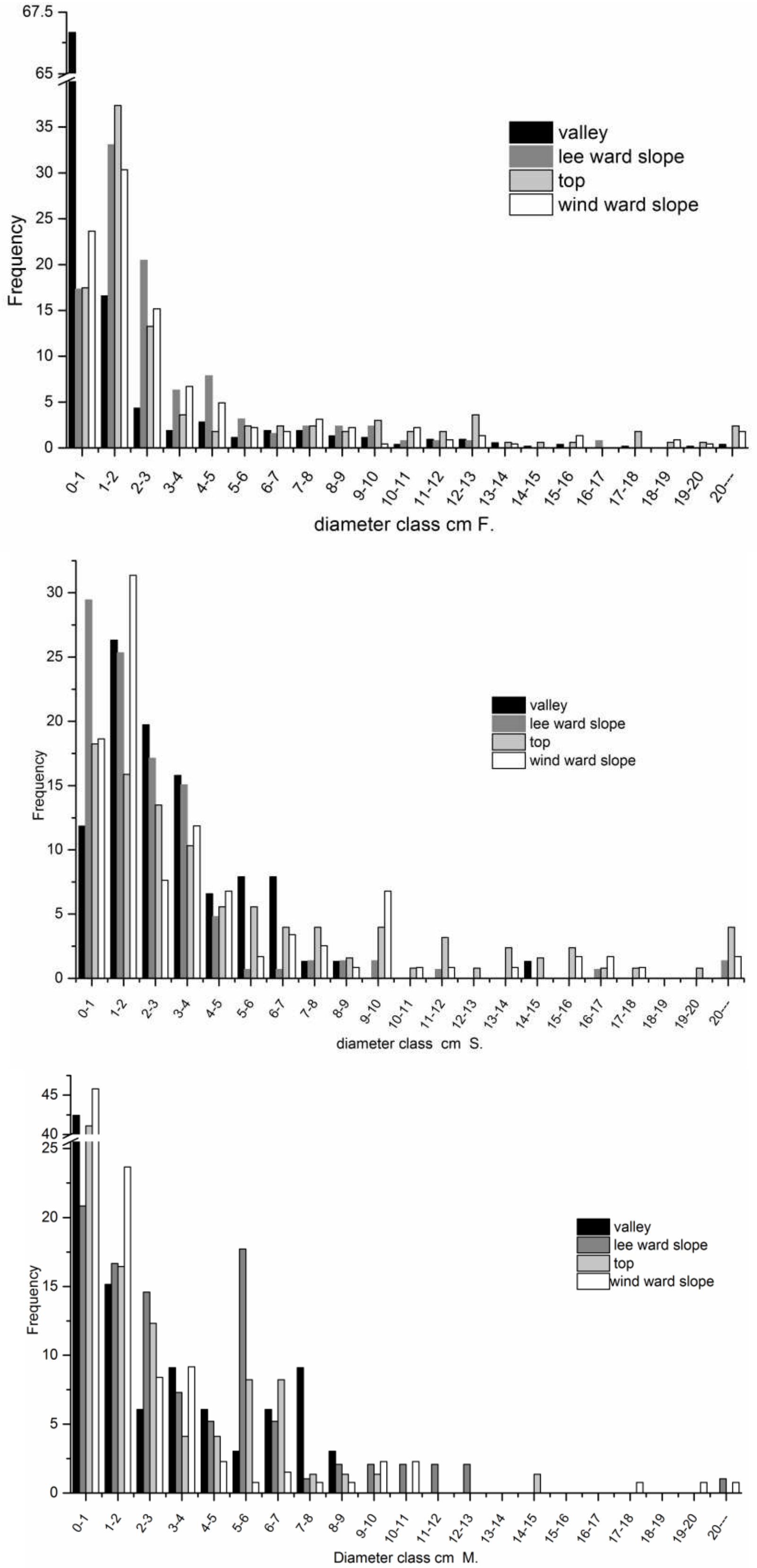

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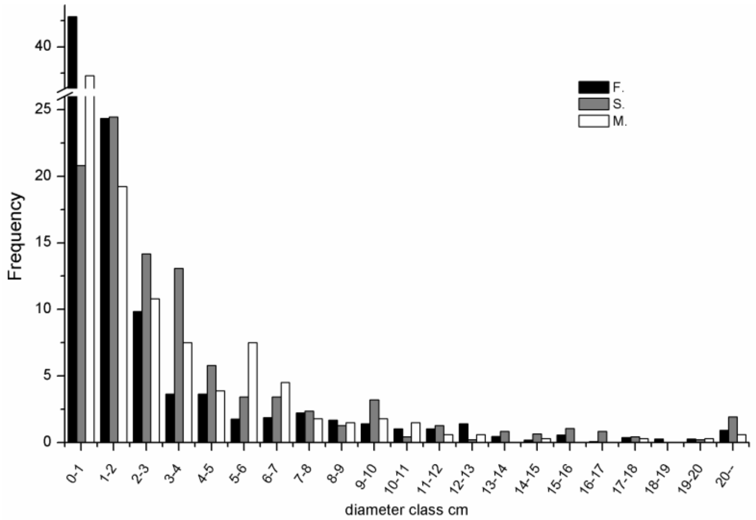

Figure 5. *Basal diameter structure of Haloxylon populations in different habitats (1class≤1cm;1≤2class≤2cm;2≤3class≤3cm;3≤4≤4cm;4≤5 class≤5cm;5≤6 class≤6cm; 6≤7 class≤7cm;7≤8 class≤8cm;8≤9 class≤9cm;9≤10 class≤10cm;10≤11 class≤11cm;11≤12 class≤12cm;12≤13 class≤13cm;13≤14 class≤14cm;14≤15 class≤15cm;15≤1 class 6≤16cm;16≤17 class≤17cm;17≤18 class≤18cm;18≤19 class≤19cm;19≤20 class≤20cm;21 class≥20cm.)*

The height structure of Haloxylon populations in different habitats

The distribution of plant heights varied among Haloxylon populations from different transects and locations on the dunes (*Fig. 6*). As with basal diameters, height distributions were also positively skewed (“reverse J” shape) across all zones, with the distributions generally peaking at 0-50cm, except for on dune tops and windward slopes in F and in dune valleys in S, where the first peak occurred at 50-100cm. Some distributions had second peaks at 150-250cm. First peaks were generally more pronounced than second peaks, except for in the S zone. In the F transect, there was a single, large peak in the first height class, a different pattern than for the other dune habitats. In the S zone, there were two distinctive peaks. In the M zone, the distribution of plant heights varied across dune sample locations, but all peaked at a height of 50cm. There was a second peak at the height of 100cm (frequency decreased from dune valleys, to tops, to leeward slopes, to windward slopes) showing that all Haloxylon populations required similar environmental conditions for growth and development. In addition, field surveys found that individuals with a height of 0-20cm were seedlings, and the proportion of seedlings was highest in populations growing at the bottom of fixed dunes (versus other population types). The population structure of Haloxylon in fixed dunes indicated a growing population. However, excluding the plants in the 0-50cm class in F, populations of Haloxylon had an almost symmetric height distribution, with the largest trees being more than 600cm tall and the smallest individuals less than 5 cm. In S and M, Haloxylon height distributions were irregular or tended towards an uneven shape.

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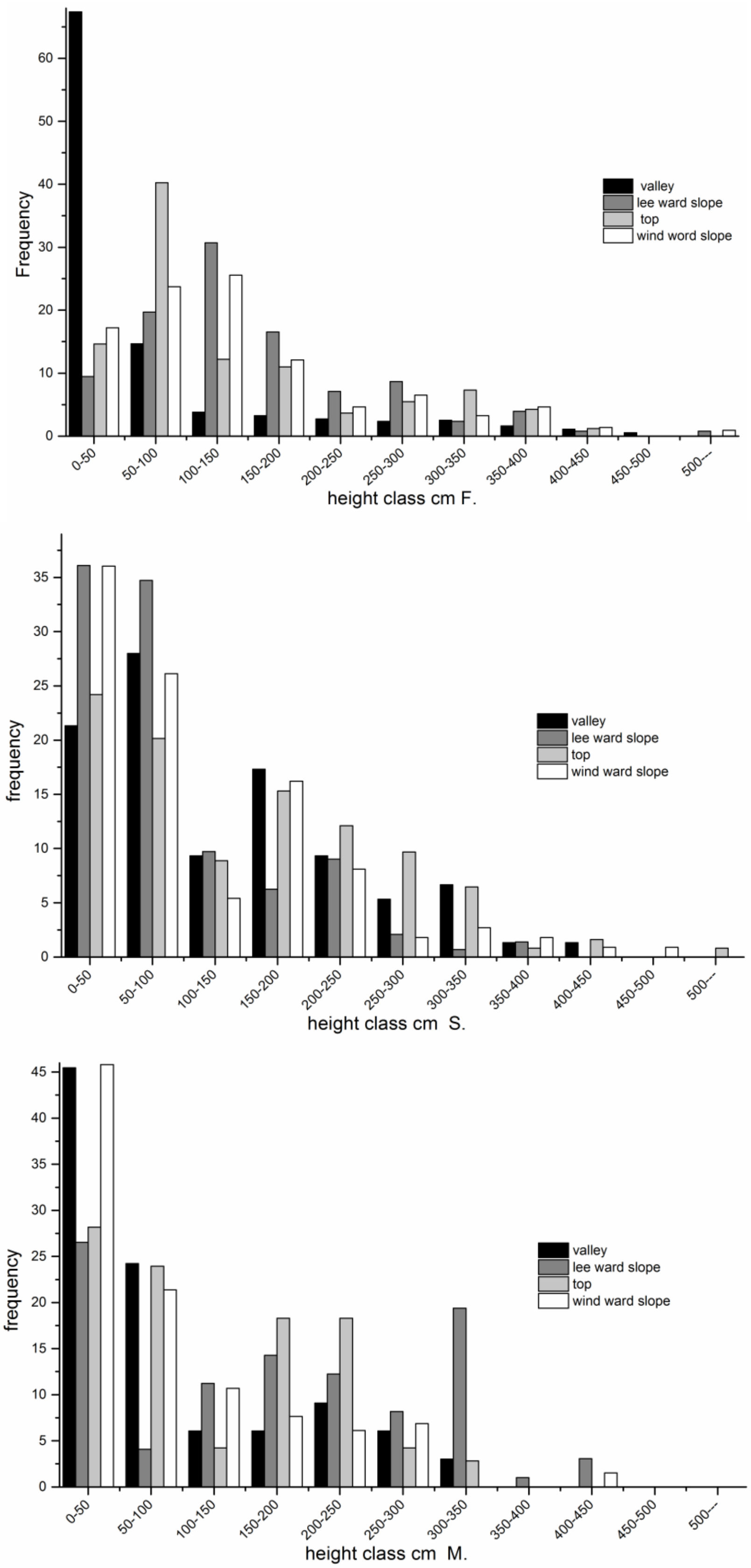
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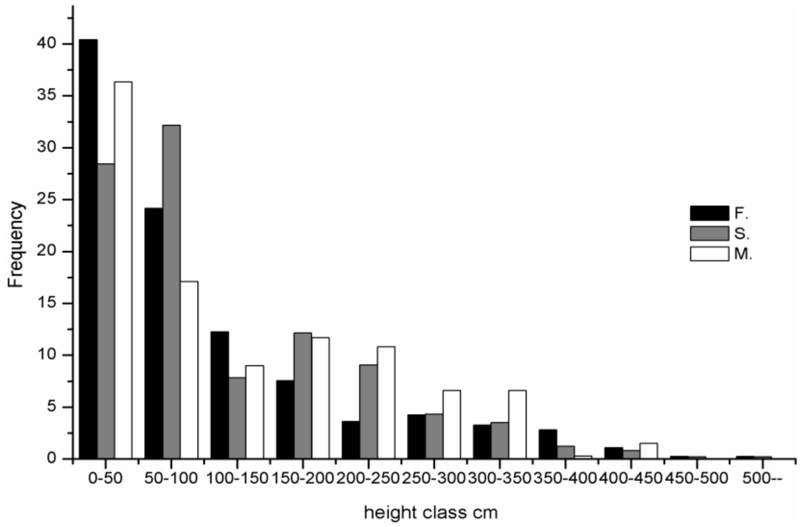

Figure 6. *Plant height structure of Haloxylon populations in different habitats (1class≤50cm;50≤2class≤100cm;100≤3class≤150cm;150≤4class≤200cm;200≤5class≤250cm;250≤6class≤300cm;300≤7class≤350cm;350≤8class≤400cm;400≤9class≤450cm;450≤10class≤500cm; 11class≥500cm.)*

Spatial distribution analysis for Haloxylon populations in different habitats

The spatial distributions of Haloxylon populations in different zones and positions on the dunes were analyzed using aggregation indices, including indicators such as the I index, the patchiness index M*/M, the Cassie index $CA=(S^2-X)/X^2$, the diffusion coefficient C and the K index (*Table 3*) .When I<1, individuals are uniformly distributed in space; when I = 1, they are randomly distributed; and when I>1, they are clumped (an aggregated distribution). When the patchiness index M*/M=1, the population has a random distribution; when M*/M>1, it is clumped; and when M*/M <1, it is uniform. When CA<0, it is a uniform distribution; when CA=0, it is random; and when CA>0, it is clumped. The diffusion coefficient $C=S^2/X$ is used to test whether a population is randomly distributed in space or not; when C=1, it is random; when C<1, it is clumped and when C>1, individuals are spatially aggregated. When the negative binomial distribution index, $K=X^2/(S^2-X)$, is K<0, it is a uniform distribution; when K>0, it is clumped; and when K tends to +∞, it is random.

The index calculations showed that all 60 Haloxylon sample populations (in the 10m× 10m-20m×20m quadrats) had clustered distributions. The diffusion coefficient C is known to be a reliable indicator of the spatial distribution of individuals within a population; it is used to test if there is any deviation from a stochastic distribution(Yan et al., 2012). The larger the C value is, the higher the degree of clustering within the population, and vice versa. As shown in *Table 3*, the C values of Haloxylon populations in semi-fixed and movable dunes were generally large, and the highest C value, of 741.73, was found for populations at the top of the semi-fixed dune, whereas the minimum value of 4.99 was found at the top of fixed dunes. Fixed dune populations had a lower degree of clustering. The mean M* in *Table 3* is the average number of neighbors for each Haloxylon individual in a one hectare area. M* was greatest at the bottom of the fixed dune, and on the west slope of the moveable dune, consistent with patterns in plant density (see *Table 2*). However, the highest percent cover values for the movable dune occurred not on the west but on the east slope, perhaps due to the

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smaller basal diameters of Haloxylon on the west slope. These results also indicate that percent cover may not be equatable with density, because of the impact of the tree crown.

Table 3. *Spatial distribution of Haloxylon populations in different habitats*

Terrain	Indicators	I	M*/M	Ca	C	K	The result
Fixed sand dunes	Valley	57.04	1.009	0.0093	58.04	107.5	Claster
	East side	14.48	1.004	0.0046	15.48	219.18	Claster
	Top	3.00	1.001	0.0012	4.99	821.56	Claster
	West side	85.96	1.02	0.023	86.96	43.42	Claster
Semi fixed snd dunes	Bottom	740.73	3.279	2.2792	741.73	0.43	Claster
	East side	153.67	1.329	0.3293	154.67	3.03	Claster
	Top	43.13	1.081	0.0815	44.13	12.26	Claster
	West side	155.19	1.284	0.2828	156.19	3.51	Claster
Mobile sand dunes	Bottom	286.1	1.726	0.727	287.1	1.37	Claster
	East side	133.8	1.185	0.186	134.8	5.38	Claster
	Top	152.2	1.25	0.25	153.2	3.99	Claster
	West side	334.4	1.415	0.415	335.4	2.4	Claster

Conclusions and discussion

Soil organic matter, texture and edaphic horizons are the most important properties of a site with regard to water availability of the soils and the differential response of plants (Troeh and Thompson, 2005). In this study, we only considered the surface edaphic horizon, as this is the one that interacts most actively with plants in the desert. Here, soil moisture and organic matter content decreased with altitude and differed significantly among three phytogeographic zones from south to central Gurbantunggut desert. Differences in soil organic matter, moisture and texture among the study geomorphological positions on dunes within the same zone (i.e. dune bottom, top or east/west slopes), produced differences in Haloxylon population characteristics. Hence, the distribution patterns and characteristics of Haloxylon populations may serve as relevant barometers of an ecosystem's sensitivity or resilience to external (environmental and anthropogenic) disturbances, although Haloxylon spp. have exceptionally broad ecological tolerances, being the main woody species in central Asian desert ecosystems (Li, 2013a). Haloxylon plays an important role in maintaining the stability of the entire desert ecosystem (Huang et al., 2009) and is considered a dominant and keystone species. In the 1970's, anthropogenic disturbance resulted in reductions in Haloxylon populations (Si, 2011), which also fed back to influence the environment, especially in certain edaphic characteristics. The mutual feedbacks between vegetation and the environment are of relevance to ecohydrological processes and to sand-binding vegetation systems in sandy desert regions of China (Li, 2013b). Several factors, including the bulk of seed rain occurring near established individuals (Shi et al., 2010) and the increase in soil nutrients and soil moisture (via hydraulic lift in plant roots) around mature plants (Li, 2007; Lamusa et al., 2008; Yuan et al., 2012) promote regeneration (i.e. most seedlings occur around mature plants, hence the higher the density of Haloxylon, the higher the recruit density).

Quantification in study plots indicated a significant decrease in Haloxylon density from the south to the central desert and a high correspondence between plant height and diameter, which both followed asymmetric distributions ("reverse J" shaped) in all

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populations. The diameter and height distributions across the three desert zones (*Fig.7*) clearly indicated growing populations, as young plants accounted for the highest proportion of individuals, with progressively fewer individuals in each larger size class. Tree density in the valleys between dunes was higher than in any other location on the dune in F, but this pattern was not found in the other zones. Few recruits were found in the central desert, but their density increased from the central to the south Gurbantunggut desert. In the drier M and S zones, trees were distinctly more scattered suggesting lower overall tree biomass, an impression confirmed in *Figure 7*, where there was less variation in tree size (fewer size classes) in M and S compared to F. These population patterns and trait distributions are consistent with previous studies (Song et al., 2011; Qian et al., 2007) that found that biomass is lower in the central desert. The availability of soil moisture and total precipitation limit plant density (Zhu and Jia, 2011). Previous studies have indicated that self-thinning processes occurred in Haloxylon plantations after 10 years of growth (Chang et al., 2008) and that plant survival decreased over time. Here, the abundance of Haloxylon trees also decreased with size (*Fig. 7*). In the plantation study, trees had access to ground water for the first 20 years, and yet there were still high mortality rates. The ground water table may perhaps have been too low to support normal physiological activity, or this mortality may have been observed for other reasons(Zeng et al., 2012; Zhang, 2010).

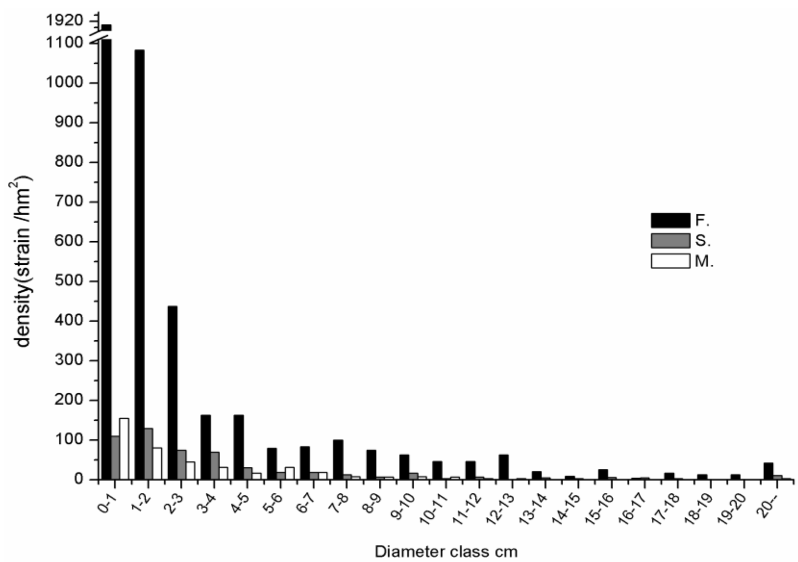

Figure 7. *The distribution of plant diameters across the three zones*

The biomass of Haloxylon individuals decreased towards the central desert These results suggest that Haloxylon is adapted to the inner desert drought conditions. Dropping assimilating branches as needed can act to maintain a balance between water supply deficits and water consumption of the assimilating organs; this morphological adjustment can reduce seedling mortality effectively. Greater allocation of photosynthate towards roots can also ensure seedling survival and a quick recovery of growth when adequate water is restored (Tian et al., 2014). In the M zone, plant stems are quickly buried by sand leading to a thin plant shape and proliferation in the number of stems (ramification below 0.5m) compared to other zones (Gai et al., 2008). New recruits were mainly found growing around mature individuals, likely because this is

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where the bulk of the seed rain occurs (Shi et al., 2010), where soil nutrients are most abundant, and where soil moisture is highest owing to hydraulic lift in plant roots (Li, 2007; Lamusa et al., 2008; Yuan et al., 2012). Hence, populations were distributed along an environmental gradient characterized by variation in water availability and soil fertility. The spatial structure of Haloxylon populations indicated clustered distributions in all zones, with recruits strongly clustered in the fixed sand dunes. Haloxylon species have a good ability to tolerate broad climatic and habitat fluctuations.

Our results illustrate that, apart from the soil physicochemical properties, climatic gradients may also have significant impacts on Haloxylon morphological traits and population structure, but that responses to climatic gradients vary significantly between phytogeographical zones. The population structure and basic characteristics of Haloxylon populations studied here resemble those found in other investigations, where a weak natural regeneration of the species in the central dessert was also observed (Si, 2011; Huang et al., 2009). Based on demographic behavior, we concluded that populations are growing in abundance in all three zones; however, the density of individuals in the M zone is too low for long-term persistence, and hence it is still a species of concern; Tree morphological traits (height, diameter, number of stems) vary according to the phytogeographical zones and change over time; and the spatial patterns of Haloxylon individuals in natural stands suggest varying degrees of aggregation.

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RECONSTRUCTION OF THE PALEOTEMPERATURE IN THE SOUTHERN MARGIN OF THE TAKLIMAKAN DESERT BASED ON CARBON ISOTOPE DISCRIMINATION OF TAMARIX LEAVES

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Abstract. Environmental factors affect carbon isotope discrimination in plants. Therefore, carbon isotope discrimination records a wealth of environmental information, such as temperature, precipitation etc., and the predominant factor's influence is significant. Based on this hypothesis, we determined the carbon isotope composition of Tamarix growing in the southern margin of the Taklimakan Desert where temperature and water are key growth-limiting factors. Mean annual temperature (MAT) showed a significant impact on variation in carbon isotope discrimination value (DS), but precipitation not. In multiple stepwise regression analysis, MAT was the first and the only variable selected into the prediction model of DS against MAT and precipitation, indicating that the effect of temperature on carbon isotope discrimination was predominant. The correlation between DS and the variation rate of precipitation in previous year shows that precipitation, as a major factor in arid regions, exerts a certain "lag effect" on the growth of Tamarix. The reconstructed MAT regression model was established according to the DS with the least squares method, the result bolster the argument that the planet's temperatures have shifted significantly in the last half-century.
Keywords: *Tamarix cone sediment laminae; temperature; precipitation; climatic factor; regression model*

Introduction

Plant carbon isotope discrimination in photosynthesis is closely associated with physiological responses including stomata conductance, assimilation rate, altered C:N allocation to carboxylation, and leaf structure (Seibt et al., 2008; Liu et al., 2014). It may be affected by many environmental factors such as temperature, moisture, altitude, latitude, longitude, solar radiation, air pressure, and atmospheric CO_2 concentration (Wang et al., 2003; Treydte et al., 2007). Based on Farquhar's research (1982), the fundamental mechanism is that these factors can control directly or indirectly the ratio of the intercellular CO_2 concentration (ci) to the ambientCO_2 concentration (ca) to affect plant carbon isotope discrimination.

Some studies have reported that precipitation has a positive influence on plant carbon isotope discrimination and altitude a negative, but that of the temperature is varied (e.g.,

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Körner et al., 1988, 1991; Morecroft and Woodward, 1996; Wang et al., 2008, 2013; Diefendorf et al., 2010; Kohn, 2010; Xu et al., 2015) inferred that the effect of altitude, longitude and latitude on plant carbon isotope discrimination could be expressed mainly in the effects of temperature and precipitation. Some researchers believed that the effects of solar radiation and air pressure are rather small compared to temperature and/or precipitation (Körner et al., 1988, 1991; Sparks and Ehleringer, 1997; Wang et al., 2008). Temperature and precipitation are considered as two fundamental influential factors.

Since plant carbon isotope discrimination is closely related to its performance, in which the key growth-limiting factors play a significant role. It has been suggested that the key growth-limiting factor is also the predominant factor affecting plant carbon isotope discrimination (Winter et al., 1982; McCarroll and Loader, 2004). Thus carbon isotope discrimination records the information of the environment when and where the plant grows, is a good carrier which can be used to reconstruct the factors of climate and the environment in history (Wei et al., 2005).

In this study, Tamarix leaf carbon isotope composition (δ13C) were investigated in the southern margin of the Taklimakan Desert (TDSM), the data of temperature and precipitation of 1960-2011 was obtained from the Cele Meteorological Station, which is closest to and the same altitude with the sampling site (*Figure 1*), the correlations was analyzed between the plant carbon isotope discrimination (DS) and temperature and precipitation. The objective was to assess whether or not temperature and precipitation can exert a dominant impact on the growth of Tamarix and reconstruct them.

Material and method

Study area

The area being studied is located in Cele Oasis, which belongs to Hotian Prefecture of Xinjiang Uygur Autonomous Region, China, in TDSM. The Cele Oasis is one of hundreds of oases, large or small, such as Hotian Oasis, Yutian Oasis and Minfeng Oasis. It lies on the transition zone between the Taklimakan Desert and Kunlun Mountains, the upstream area of Celeriver's alluvial fan, and belongs to continental arid desert climate, with an annual average temperature of 12.13 °C and frequent wind sandy activities. According to the data gathered from Cele Meteorological Station, the annual precipitation is only 38.4mm, 50% to 70% of which concentrates in the period from June to September; but the annual average evaporation can reach to 2500 ~ 3400mm. The zonal vegetation is mainly made up of the xerophilous, super-xerophytic shrubs and semi-shrubs, as well as some alkali-saline-tolerant perennial herbs.

On both sides of riverbanks, delta areas, ancient riverbanks and local lowlands of the rivers, there are scattered Tamarixes, taking carbon from the air and oxygen and hydrogen from deep soil, standing fixed in the desert area often for centuries. Their unique physical characteristics enable them to survive in the arid, high salt desert regions. The longtime collaboration between wind sandy and Tamarix has formed Tamarix cones, a unique bio-geographical type, composed of alternate layers of sand and Tamarix twigs and leaves (Xia et al., 2004).

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Sample collection

From June 18th to 20th in 2011, in Damagou of Cele County (*Fig. 1*), a typical Tamarix cone was chosen. From top to bottom, the samples were collected layer by layer. If the sedimentary vein was not clear, they were collected accorded to the average thickness. The sample site is located at37.09 ° N, 81.08 ° E, 1318m above sea level; 151 samples of sedimentary veins of Tamarix cones were collected. The depth of the profile is about 4.5m.

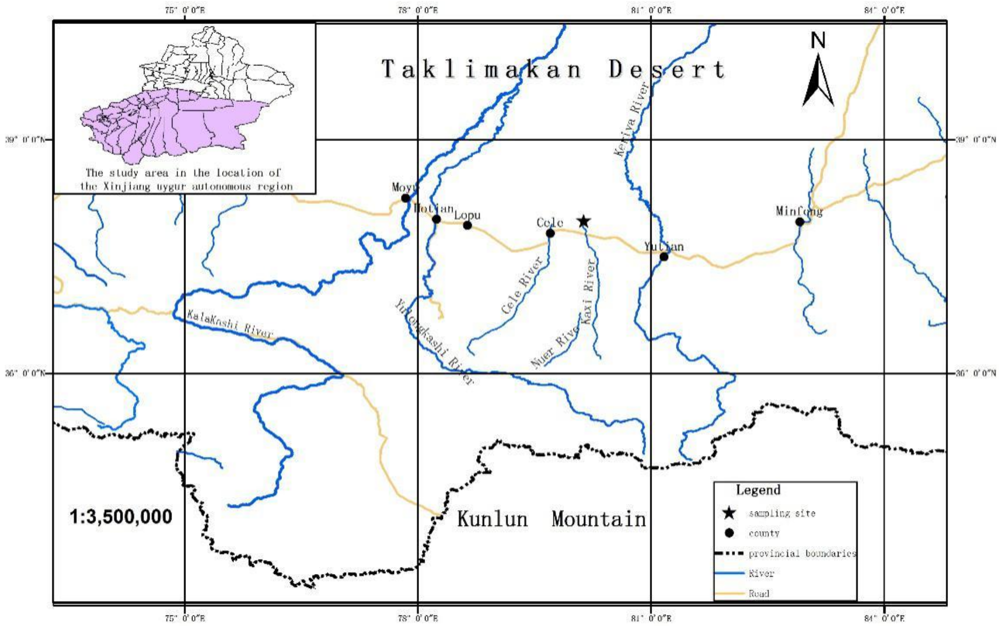

Figure 1. *Location map of sampling site in Damagou of Cele County*

Data measurement

The age sequence of δ13C values was identified according to overall consideration of the age data derived from 210Pb, 137Cs dating methods, which were done in Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences (CAS), combined with the calculation of14C dating, which was done by the Accelerator Mass Spectrometry Laboratory in Beijing University, the rate of Tamarix cone sedimentation and the thickness of the each sedimentary layer (Zhao et al., 2015).

The δ13C values of all samples were measured in Nanjing Institute of Geography and Limnology CAS, on the base of VPDB (the standard error of 0.1‰). The test steps are as follow: after the collected leaves were well pretreated, the obtained alpha cellulose was burned by Flash EA1112 Eelemental Analyzer; the combusted gas was sent into Delta Plus Advantage Isotope Mass Spectrometer to measure organic carbon isotope ratio of the sample (13C/12C).

The carbon isotope discrimination (DS) of Tamarix leaves is obtained by following formula based on Farquhar et al. (1989):

$$DS = \frac{\left(\delta^{13}C_{ate} - \delta^{13}C_t\right)}{\left(1 + \delta^{13}C_t / 1000\right)} \approx \delta^{13}C_{ate} - \delta^{13}C_t \qquad \text{(Eq.1)}$$

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In Eq. 1, the carbon isotope ratio of atmospheric CO_2, which were measured from ice core bubbles or plant δ13C value (Friedli et al., 1986; Leavitt and Long, 1989; McCarroll and Loader, 2004), $\delta^{13}C_t$ is the carbon isotope ratio of Tamarix leaves.

Statistical analysis

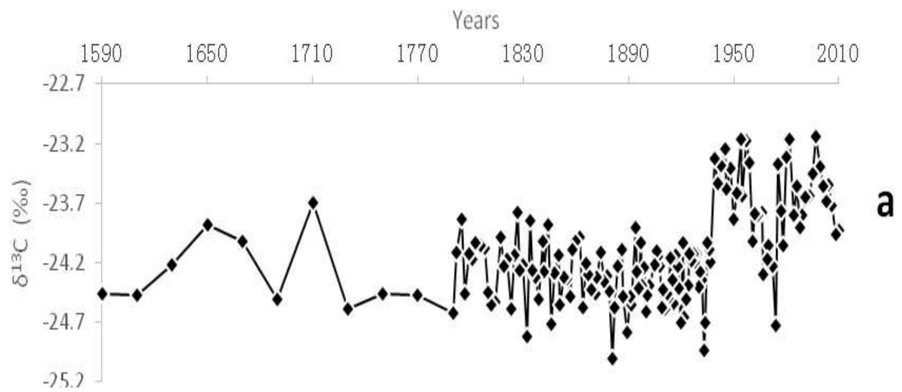
Descriptive statistical analysis was carried out on δ13C and DS, then a bivariate correlation analysis was performed to examine the links between plant DS and temperature and precipitation. Considering the existence of potential interactions between temperature and precipitation, partial correlation analysis, in which temperature and precipitation were separately controlled, was applied to describe the actual links between Tamarix leaf DS and temperature and precipitation. Regression analysis was used to constrain the influences of temperature and precipitation on Tamarix leaf DS. The regression models were established according to the Tamarix leaf DS with least square fitting method to reconstruct the history climate information. All statistical analysis was performed using IBM SPSS Statistics 19.0 (IBM Corporation, New York, NY, USA).

Results

The $\delta^{13}C$ and DS

The $\delta^{13}C$ values of the sample site were established in the period from 1590 to 2010 (*Figure 2a*). The time span of this sampling sequence is 420 years. The $\delta^{13}C$ values are within the range from -25.008‰ to -23.138‰, the average $\delta^{13}C$ value is -24.137‰. In the period from 1790 to 1937, the δ13C value is lower and fluctuates frequently. In 1790, 1832, 1881 and 1933, the relatively low values appear, which are -24.625‰, -24.824‰, - 25.008‰ and - 24.938‰ respectively, and in 1881 the minimum value of the data series appears. There is a leap in 1939, 3.6% higher than that in 1937, and the $\delta^{13}C$ value is greater and more dynamic till 2010, with the emergence of a maximum value of -23.138‰ in 1997. In 1982, 1957 and 1954, the δ13C value is higher, but in 1974 it appears as a relatively lower value for this period.

The carbon isotope discrimination values (DS) were obtained by formula (1) (*Figure 2b*). The values are within the range from 15.228‰ to 18.458‰, the average DS value is 17.372, and show a downward trend.



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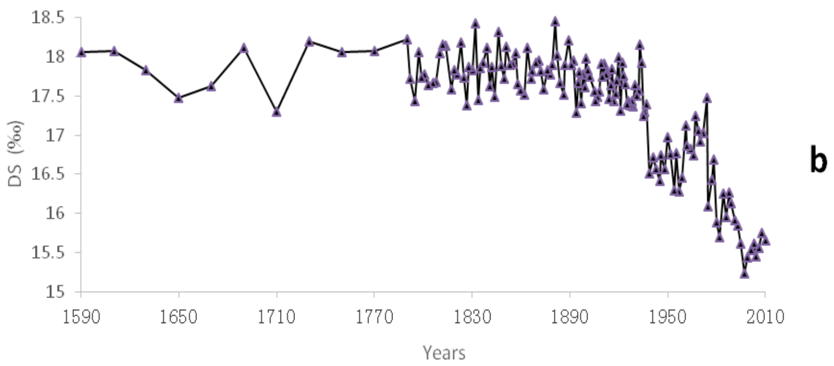

Figure 1. *The observed sequences of the δ13C values (a) and the carbon isotope discrimination values (b)*

Correlations analysis

The correlation between the $\delta^{13}C$ values and temperature and precipitation and that between DS and temperature and precipitation are showed in *Table 1*. Considering that the response of carbon isotope and carbon isotope discrimination to environmental factors may be dependent on Tamarix functional type, the influence of temperature and precipitation on δ13C and DS were analyzed separately.

There was a positive correlation between temperature and δ13C only in February (r=0.510, p=0.005). The DS values were correlated with the temperature in February and growing season, and significantly with mean annual temperature (MAT) (r=-0.761, p=0.000).

The $\delta^{13}C$ values were correlated with precipitation only in February (r=0.488, p=0.007) of that year, and the DS values were not associated with precipitation either in that year or previous year. But the precipitation changes rate of previous year (PCR) (i.e., the natural logarithm of the precipitation in the previous year) were correlated with DS (r = -0.376, p = 0.044) (shown as *Table 2*).

Table 1. *The correlation between the δ13C, DS values and temperature and precipitation (N=29)*

	Between $\delta^{13}C$ and Temperature		Between DS and Temperature		Between $\delta^{13}C$ and Precipitation		Between DS and Precipitation	
	Correlation	Sig.	Correlation	Sig.	Correlation	Sig.	Correlation	Sig.
Jan	0.312	0.1	-0.311	0.101	-0.362	0.053	0.117	0.545
Feb	**0.510****	**0.005**	**-0.445***	**0.015**	**-0.488****	**0.007**	0.2	0.297
Mar	0.109	0.575	-0.305	0.108	-0.026	0.894	-0.224	0.243
Apr	-0.143	0.46	-0.016	0.934	-0.07	0.718	0.142	0.462
May	-0.052	0.788	-0.181	0.346	-0.209	0.276	0.13	0.500
Jun	0.149	0.442	-0.229	0.232	-0.074	0.703	-0.114	0.556
Jul	0.25	0.19	**-0.450***	**0.014**	-0.163	0.399	0.198	0.304
Aug	0.176	0.361	**-0.432***	**0.019**	0.101	0.602	-0.07	0.717
Sep	0.167	0.387	**-0.542****	**0.002**	0.191	0.322	-0.096	0.621
Oct	0.266	0.164	**-0.525****	**0.003**	0.036	0.851	-0.116	0.547
Nov	-0.021	0.916	-0.305	0.108	0.029	0.881	0.118	0.541
Dec	-0.137	0.479	-0.183	0.341	-0.174	0.366	0.019	0.921
Annual	.427*	0.021	**-0.761****	**0.000**	0.081	0.674	0.041	0.833

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Table 2. *The correlation between DS and precipitation of previous year (N=29)*

	Precipitation	The change rate of precipitation
Pearson Correlation	-0.335	-0.376*
Significant (bilateral)	0.075	0.044

Multiple stepwise regression analysis model and reconstruction model

In view of the correlations between DS and MAT and PCR, multiple stepwise regression analysis was applied. The result reveal that MAT was the only variable entered in the stepwise regression model of DS ($DS = 23.645 - 0.608MAT$, $R^2 = 0.580$, $\bar{R}^2 = 0.564$, $p = 0.000$). So the Tamarix leaf DS values (DSt) can be used as a regression factor to reconstruct MAT ($\hat{T}_t$), the MAT reconstruction equation is as follow:

$$\begin{aligned} \hat{T}_t &= 27.668 - 0.953 * DS_t \\ se \quad & (2.534) \qquad (0.156) \\ p-value \quad & [0.000] \qquad [0.000] \\ R^2 &= 0.580, \quad \bar{R}^2 = 0.564 \end{aligned} \qquad \text{(Eq.2)}$$

The curve of reconstructed temperature were shown in *Figure 3*.

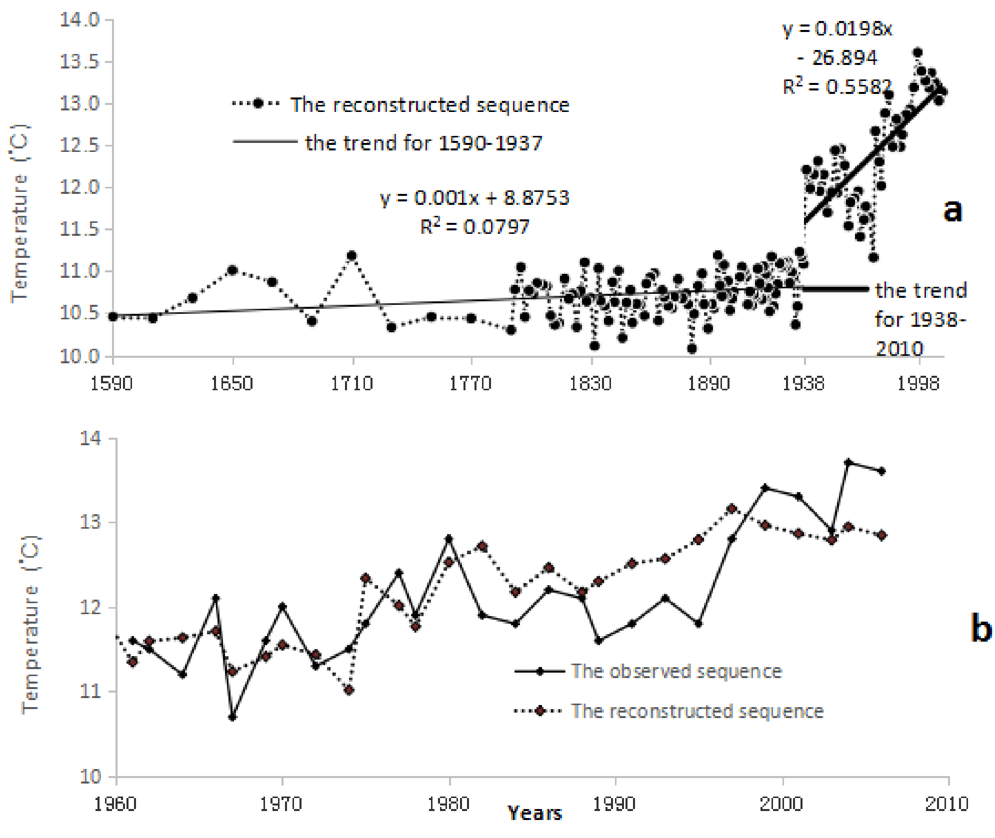

Figure 3. *(a)The reconstructed temperature from 1590-2010 and (b)the comparison between the observed and reconstructed temperature from 1960-2010*

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Disscussions

Features of the δ13C values

The trend of Tamarix leaf $\delta^{13}C$ values from Cele region in TDSM is characterized by vibration and complexity. The values are very close to those in the Minfeng Andier ancient city and the Andier meadow sampling site (Sun, 2013), consistent with the results of Lop Nur region (Xia et al., 2004; Zhao et al., 2011a). The results are basically in accordance with the δ13C composition of C3 plants in arid environments (Ehleringer and Monson, 1993).

This results show that the difference of Tamarix leaf $\delta^{13}C$ values in different sites of TDSM is tiny. The δ13C values show small regional differences because the sampling sites are geographically different, which proves that the variation of $\delta^{13}C$ value of Tamarix leaves in arid regions is stable within species and regions. In addition, compared with the extremely arid Lop Nor region, the hinterland of the desert, the climate of Cele region in TDSM is relatively humid. Therefore, the Tamarix leaf $\delta^{13}C$ values show a gradual decrease with the improvement of water conditions. This conclusion is similar to that of some conclusions (Liu et al., 1997; Sun et al., 2003; Yun et al., 2010; Zhao, 2012).

Effect of temperature on Tamarix

The correlation between temperature and DS is more significant than that with δ13C, and the DS sequence is correlated with the temperature in growing season and MAT negatively and significantly (*Table 1*).Combining the results of stepwise regression, we believe that temperature has exerted the key influence on carbon isotope discrimination of the Tamarix growing in arid area, and the DS decline 0.608 for each degree rise in MAT.

Temperature is one of the most important factors that control plant growth and certain physiological processes related to plant gas exchange activity. A decline temperature usually results in a reduction in enzyme activity and photosynthetic rate (Beerling, 1994), thus leading to decreased CO_2 assimilation and a lower growth rate as a consequence. An increase in DS with decreasing temperature is therefore expected and has been observed in most of the studies on the influence of temperature on carbon isotope discrimination (e.g., McCarroll and Loader, 2004; Treydte et al., 2007; Wang et al., 2013).

Our results are consistent with these studies and suggest a decline in DS with increasing temperature. The temperature influences the growth of Tamarix directly, and the higher mean annual temperature of the growing season is more beneficial to Tamarix. That shows an agreement with some researchers (Wang et al., 2003; Liu et al., 2007; Shen and Chen, 2000; Zhao et al., 2011b).

Effect of precipitation on Tamarix

Numerous studies have reported the influence of water availability on plant carbon isotope discrimination (e.g., Wang et al., 2005, 2008; Diefendorf et al., 2010; Kohn, 2010), and a positive correlation between DS and water availability has been observed on most occasions. The mechanism of water availability on plant carbon isotope discrimination is that the plants would close their stomata to reduce water loss when moisture decreases, resulting in a lower ci/ca ratio and less negative δ13C values.

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In this study, the δ13C and DS values are with little dependence on contemporary precipitation but in February (r= -0.448, p=0.007) (*Table 1*). At the same time, correlation analysis (*Table 2*) is made between DS sequence and the precipitation in the previous year and the precipitation changes rate of previous year (i.e., the natural logarithm of the precipitation in the previous year). The correlation coefficients are -0.335 and -0.376 (p=0.044).

The effect of precipitation on the growth of Tamarix is weaker. Preliminary analysis suggests that in more precipitation year, more snow and ice were deposited on the mountains than normal year, which may result in the larger quantity of underground water availability in growth period of Tamarix in the next year, and consequently promoted the photosynthetic rate and Tamarix growth.

This further proves the "lag effect" of precipitation (Leavitt and Long, 1989) on Tamarix growth. The precipitation is an important factor influencing the growth of Tamarix in arid region, but not directly, and the mechanism is complex.

Analysis of reconstructed temperature in past 400a

The temperature from 1590 to 2010 were reconstructed based on DS (*Figure 2a*),could be divided into sub-periods by the year 1937 based on the trend.

The first period (1590 - 1937): It is a 347-year time span. The trend line slanting rate of reconstructed temperature is 0.001, the temperature were more stable with a slighter fluctuation.

The second phase (1938 - 2010): In the 72 years, the temperature underwent a remarkable increase, the trend line slanting rate is 0.0198, and its range of oscillation was extended. It can be defined as the modern warm and wet period.

The explained variance of reconstructed mean annual temperature is 56.4%, which is reliable. The results show a tendency toward warming, and the change will be more frequent and more intense, which reflects the characteristics of global climate change.

Conclusion

The present study conducted an investigation of Tamarix leaf δ13C in TDSM and analyzed the influence of temperature and precipitation on the variation in DS. Temperature, the key growth-limiting factor for Tamarix, was found a significant influence on DS, but the influence of precipitation was relatively weak. Future study should consider on water availability in relation to Tamarix carbon isotope discrimination. Ecosystems in TDSM are fragile and sensitive to climate change. With the rising temperature, more precipitation will be expected, which may have enormous impacts on productivity and stability of ecosystems in the future.

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IDENTIFYING THE SOURCE OF POLLUTANTS IN MALACCA RIVER USING GIS APPROACH

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Abstract. The study was conducted to determine the dominant source of pollutants in Malacca River using the combined methods of water sampling and GIS approach. The study was conducted in 9 sampling stations based on Malacca River sub-basins. The result of WQI indicated that station 4 and 5 are polluted; station 8 and 9 are clean; and other stations are slightly polluted. PCA identified several pollutant sources, namely agricultural, residential, industrial, animal husbandry activities, as well as sewage treatment plants. Applied GIS technique detected several areas as hotspots pollutants sources, namely agricultural activities in station 5; residential activities in station 1, 2, 5, 6, and 7; industrial activities in station 3, 4, 5, and 7; animal husbandry in station 5 and some scatterings in station 1 to 4; as well as sewage treatment plant in moderate hotspot area between station 5 and 6, respectively. Besides the recommendation to reduce the river water pollution through the control of pollutants source, this study provides crucial information for the identification of problematic areas and spatial database of Malacca River for better understanding and management of river water quality in the future, as well as a reference for future land use and urban design development purposes.
Keywords: *WQI, PCA, hotspot analysis, spatial database*

Introduction

River water pollution has received great attention in recent years and continues to receive serious concern throughout the world. Water quality deterioration is primarily connected to the subject of population growth and city expansion. This is a threatening factor to human and ecological health, drinking water availability, and furthermore to the economic development (Houser and Richardson, 2015; Morse and Wolheim, 2014; Li and Zhang, 2010). According to Iscen et al. (2008), surface water is easily exposed to pollution due to its - accessibility to wastewater disposal. Water quality impairment resulted from anthropogenic inputs (e.g. municipal and industrial wastewater discharges, agricultural runoff) and natural processes such as chemical weathering and soil erosion (Shin et al., 2013; Singh et al., 2011; Iscen et al., 2008), contributed to the input of non-point and point source pollutants of the river (Iscen et al., 2008). Therefore, water quality assessment with geographic information system (GIS) is an important tool in identifying possible pollutant sources with the aim to prevent and control water pollution; which is crucial for sustainable water resource use with respect to ecosystem health and social development (Iscen et al., 2008; Shrestha and Kazama, 2007; Zhang, 2006).

Malaysia as an ongoing developing country in South East Asia is facing major water quality problems (DOE, 2012). Human activities that generate economic benefit for the society has indirectly deteriorate the water quality of the river (Muyibi et al., 2008). Several studies focused on the assessment of water quality indicated that unsustainable development could result in environmental damage to surrounding areas, as well as to the biodiversity of benthic macroinvertebrates (Al-Shami et al., 2010). Specifically,

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researchers have identified that wastewater that were discharged from the manufacturing and agro-based industry, domestic sewage, animal husbandry, mining activity, and surface runoff originating from land clearing and earthwork activity; could lead to water resource pollution, especially in the river (DOE, 2012; Suratman et al., 2009; Deb et al., 2008; Ebrahimpour and Mushrifah, 2008; Muyibi et al., 2008).

This situation is no stranger to the state of Malacca, which has faced serious water pollution problems that led to the death of aquatic species along the Malacca River (Sinar Harian Online, 2016; Hua, 2015; Metro Online, 2015; Daneshmend et al., 2011). Malacca State was recognized by UNESCO as a World Heritage Site in 2008 (UNESCO, 2016) and since has become a world historical tourism center for the country. This establishment is important for the economic and population growth of Malacca State. Indirectly, Malacca River may not have been exposed to the issues of river water pollution in the past. Nevertheless, the increasing number of population, uncontrolled rapid development, and extreme land use has led to the 'disruption' of Malacca River. Besides water quality assessment and monitoring, an applied GIS through hotspot analysis would assist in determining the dominant source of pollution that has greater impact to Malacca River. GIS hotspot analysis is a method that has been frequently applied in various studies in the fields of diseases (Liu et al., 2006), mortality rates (Mclaughlin and Boscoe, 2007), environmental planning, as well as the environmental sciences (Ishioka et al., 2007). For instant, Liu et al. (2006) used GIS to assess and sample the pattern of heavy metal in paddy field; and Zhang (2006) used hotspot analysis of GIS approach to identify the pollutants in urban soils in Ireland.

Several GIS analysis methods have been proposed for hotspot analysis, such as spatial scan statistics (Ishioka et al., 2007), Tango' C index (Zhang and Lin, 2006; Tango, 1995), as well as Getis's G index (Getis and Ord, 1992). These methods are often used in the field of environmental sciences, planning, and management. Hotspot analysis which is extended from Moran's I index in spatial analysis, can be classified into two categories, namely global Moran's I (Oldland, 1998; Cliff and Ord, 1981) and local Moran's I index (Zhang et al., 2008; Mclaughlin and Boscoe, 2007; Getis and Ord, 1992). Unlike the particular analysis of Moran's I which only focuses on the detection of similar value clusterings, hotspot analysis technique using G-statistic has the ability to express the high/low value clusterings (Getis and Ord, 1992). This technique of hotspot analysis is applied to this study. The objectives of the study are (1) to identify water quality status and pollution sources using relationship elements of natural origins; and (2) to determine the dominant sources of pollutants through spatial pattern analysis.

Materials and Methods

Study Area

The state of Malacca is located in the southwest of Peninsular Malaysia with the geographical coordinates of 2°23'16.08"N to 2°24'52.27"N latitude and 102°10'36.45"E to 102°29'17.68"E longitude. Malacca is divided into three districts, namely Alor Gajah, Jasin, and Malacca Central. Total catchment area of Malacca is approximately around 670 km^2 with about 80 km distance of Malacca River. The basin is formed by 13 sub-basins namely Kampung Ampang Batu Gadek sub-basin, Kampung Balai sub-basin, Kampung Batu Berendam sub-basin, Kampung Buloh China sub-basin, Kampung Cheng sub-basin, Kampung Gadek sub-basin, Kampung Harmoni Belimbing Dalam sub-basin, Kampung Kelemak sub-basin, Kampung Panchor sub-basin,

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Kampung Pulau sub-basin, Kampung Sungai Petai sub-basin, Kampung Tamah Merah sub-basin, and Kampung Tualang sub-basin (*Figure 1*). Among the 13 sub-basins, only 7 sub-basins were selected, with 9 sampling stations located alongside Malacca River.

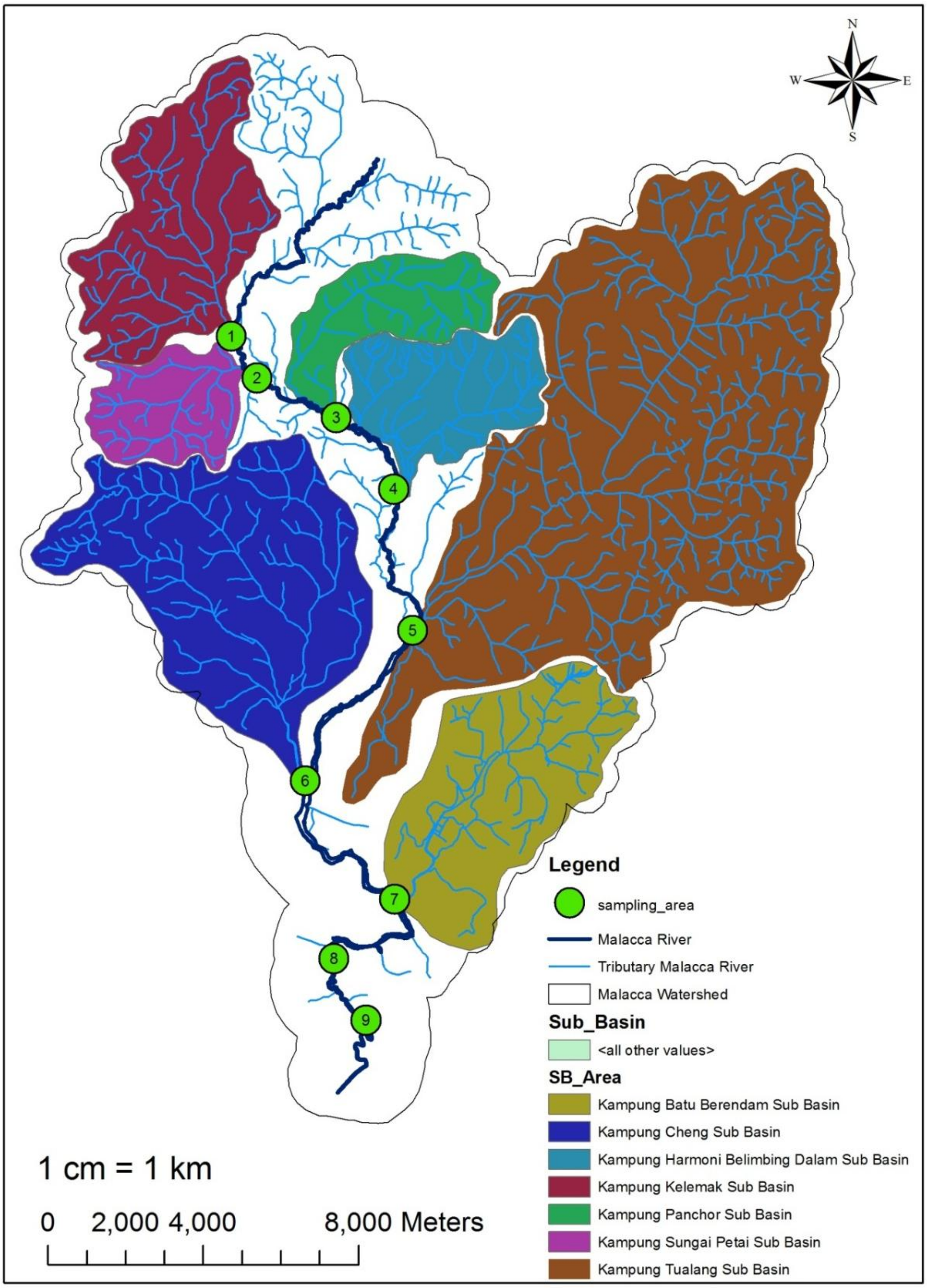

Figure 1. *Malacca River sub-basin and sampling area*

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Malacca River flows across Alor Gajah to the Malacca Central district before entering into the Straits of Malacca. Alongside Malacca River, there is a reservoir located between Alor Gajah and Malacca Central, namely Durian Tunggal Reservoir, with a catchment area of approximately 20 km^2 that act as a source of water supply for Malacca residents. The built-up area is mainly concentrated in the city center, Malacca Central, at a downstream area extending about 10 km to the west, 10 km to the east, and 20 km to the north. The urban land uses are primarily residential and commercial, while several industrial activities including high-technology and estates are located in the middle-stream and upstream areas. Most of the large-scale agricultural activities land use are located upstream.

Field Sampling

There were 9 sampling stations chosen alongside Malacca River. The locations were determined using a Global Positioning System (GPS) coordinates as shown in *Table 1* and the geographic coverage is as shown in *Figure 1*. The collection of water quality samples were carried out monthly from January to December 2015.

Table 1. *The latitude and longitude of sampling stations*

Station	Latitude	Longitude
S1	02°21’57.41”N	102°13’7.10”E
S2	02°21’30.16”N	102°13’18.20”E
S3	02°20’49.52”N	102°14’36.44”E
S4	02°19’41.70”N	102°15’27.30”E
S5	02°17’48.86”N	102°15’39.51”E
S6	02°15’46.55”N	102°14’10.72”E
S7	02°14’5.02”N	102°15’24.67”E
S8	02°13’14.33”N	102°14’35.01”E
S9	02°12’23.42”N	102°15’0.80”E

Source: Global Positioning System

The water samples were collected using ‘grab sampling’ technique in the polyethylene bottles without entrapping the air bubbles. Each bottle was labelled with the corresponding sampling station and kept at 4°C to minimize microbial activity in the water (APHA, 2005). The water samples were analyzed for physico-chemical parameters (i.e. pH, temperature, electrical conductivity (EC), salinity, turbidity, total suspended solid (TSS), dissolved solids (DS), dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), and ammoniacal-nitrogen (NH_3-N), trace metals (i.e. mercury, cadmium, chromium, arsenic, zinc, lead and iron)) and biological parameters (i.e. Escherichia coliform and total coliform). Since water sample containing colloidal or suspended particulate material could interfere with the metal analysis, the samples were immediately filtered using 0.45 µm cellulose acetate membrane filter (Whatman Milipores, Clifton, NJ) at the laboratory. The purpose of this procedure was to prevent the occurrence of clogging during analysis with spectrometry instruments and to obtain the dissolved ions for metal analysis (APHA, 2005). Then, the samples were acidified with HNO_3 to pH<2 in order to prevent precipitation of the components for trace metal analysis and to retard any biological activities (APHA, 2005).

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Water Quality Analysis

The water samples were analyzed according to the procedure of APHA (2005), meaning that pH, turbidity, EC, TDS, salinity, and DO were measured on-site. SevenGo Duo pro probe (Mettler Toledo AG) was used for the measurement of pH values, while turbidity was tested using the Handled Turbidimeter Hach 2100. Orion Star Series Portable Meter was used to measure temperature, EC, DS, salinity, and DO. Meanwhile, NH3N was analyzed using a spectrophotometer at a specific wavelength using Hach Method 8038, COD was measured using APHA 5220B open reflux technique; BOD was measured using APHA 5210B (or Hach Method 8043); and TSS was measured using the APHA 2540D method. Both E-coli and total coliform were analyzed using Membrane Filtration method based on APHA 9221B, and trace metals were analyzed using inductive coupled plasma-mass spectrometry (ICP-MS, ELAN DRC-e 6100, Perkin Elmer). For quality assurance and quality control purposes, laboratory apparatus and polyethylene bottles were washed using 5% (v/v) of nitric acid and soaked overnight to remove contaminants and traces of cleaning reagent before the collection of water samples or conducting laboratory analysis.

Each sample was analyzed in triplicate before calculating the mean value, and standard deviation (SD) of less than 20% was used as an indicator of precision for each measured parameter. All the probe meters and instruments used were calibrated prior to analysis. In all cases, the standards and blank were treated in the same way as the representative river water samples to minimize matrix interference during analysis. The recovery of samples for all target elements fell within the standard requirements (90-110%).

Data Analysis

River water quality data were analyzed using Microsoft Excel and Statistical Package for Social Sciences version 19 (SPSS 19) for descriptive analysis, water quality index (WQI) and principal component analysis (PCA); to identify the water status and pollution source between elements of origin parameters, while ArcGIS version 9.3 was used to determine the dominant source of pollutants through spatial pattern analysis.

Water Quality Index (WQI)

Healthy river should have good water quality to assist with the survival of aquatic animals. The river health level is measured using WQI, which based on several parameters that need to be assessed and monitored. Different country uses different parameter to determine the WQI, whereas the Department of Environment (DOE) Malaysia using DO, BOD, COD, NH_3N, SS, and pH in determining the WQI. Generally, DO is use to measure the amount of oxygen available in water (Juahir et al., 2011); BOD determines the strength of pollutants in term of oxygen required to stabilize the wastes or measures biodegradable waste present in water (WSDE, 2002); COD measure the amount of organic and inorganic oxydizable compound in water (Davis and McCuen, 2005); SS determines the natural pollutants and causes of turbidity in water (Mathvi and Razazi, 2005); NH_3N determine the amount of ammonia exists in water that could cause eutrophication (Wang et al., 2010); and pH are to measure the acid strength in water (Davis and McCuen, 2005). Therefore, WQI for Malacca River are determined using formula that was developed by DOE (Eq.1), which consists of different sub-indexes (SIs) calculated according to the best-fit relationship (Eq.2-7):

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$$WQI = 0.22 * SI_{DO} + 0.19 * SI_{BOD} + 0.16 * SI_{COD} + 0.15 * SI_{AN} + 0.16 * SI_{SS} + 0.12 * SI_{pH} \quad \text{(Eq.1)}$$

where WQI is water quality index; SI_{DO} is sub-index of DO; SI_{BOD} is sub-index of BOD; SI_{COD} is sub-index of COD; SI_{AN} is sub-index of NH_3N; SI_{SS} is sub-index of SS; SI_{pH} is sub-index of pH. Meanwhile, the sub-indexes (SIs) formulation is as followed (Eq.2-7);
Best-fit equations for DO sub-index:

$$SI_{DO} = \begin{cases} 0 & \text{for DO} < 8 \\ 100 & \text{for DO} > 92 \\ -0.395 + 0.030DO^2 - 0.00020DO^3 & \text{for } 8 < DO < 92 \end{cases} \quad \text{(Eq.2)}$$

Best-fit equations for BOD sub-index:

$$SI_{BOD} = \begin{cases} 100.4 - 4.23BOD & \text{for BOD} < 5 \\ 108e^{-0.055BOD} - 0.1BOD & \text{for BOD} > 5 \end{cases} \quad \text{(Eq.3)}$$

Best-fit equations for COD sub-index:

$$SI_{COD} = \begin{cases} -1.33COD + 99.1 & \text{for COD} < 20 \\ 103e^{-0.0157COD} - 0.04COD & \text{for COD} > 20 \end{cases} \quad \text{(Eq.4)}$$

Best-fit equations for AN sub-index:

$$SI_{AN} = \begin{cases} 100.5 - 105AN & \text{for AN} < 0.3 \\ 94e^{-0.573AN} - 5|AN - 2| & \text{for } 0.3 < AN < 4 \\ 0 & \text{for AN} > 4 \end{cases} \quad \text{(Eq.5)}$$

Best-fit equations for SS sub-index:

$$SI_{SS} = \begin{cases} 97.5e^{-0.00676SS} + 0.05SS & \text{for SS} < 100 \\ 71e^{-0.0016SS} - 0.015SS & \text{for } 100 < SS < 1000 \\ 0 & \text{for SS} > 1000 \end{cases} \quad \text{(Eq.6)}$$

Best-fit equations for pH sub-index:

$$SI_{pH} = \begin{cases} 17.2 - 17.2pH + 5.02pH^2 & \text{for pH} < 5.5 \\ -242 + 95.5pH - 6.67pH^2 & \text{for } 5.5 < pH < 7 \\ -181 + 82.4pH - 6.05pH^2 & \text{for } 7 < pH < 8.75 \\ 536 - 77.0pH + 2.76pH^2 & \text{for pH} > 8.75 \end{cases} \quad \text{(Eq.7)}$$

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Principal Component Analysis (PCA)

Principal component analysis was designed to convert a large dataset of original correlated variables into a smaller set of new and uncorrelated variables (i.e. principal components). The data reduction process would provide the most meaningful parameter information that can describe a whole data set with minimum loss of original information (Iscen et al., 2008). The principal components are weighted linear combinations of original variables, with the first principal component representing the largest variability of the original data set, and the second component representing the next largest variance that is orthogonal to the first component (Deb et al., 2008). In other words, PCA can be explained as follows:

$$z_{ij} = a_{i1}x_{1j} + a_{i2}x_{2j} + \ldots + a_{im}x_{mj} \qquad \text{(Eq.8)}$$

Where *z* is the component score, *a* is the component loading, *x* is the measured value of the variable, *i* is the component number, *j* is the sample number, and *m* is the total number of variables. As stated above, the general procedures used in PCA are (1) the hypothesis in an original data group is then reduced to dominant components or factors (source of variation) that influence the observed data variance; and (2) the whole data set is extracted through eigenvalues and eigenvectors from the square matrix produced by multiplying the data matrix (Aris et al., 2013). In other words, the PCA will undergo varimax rotation to produce the principal components (PCs) before determining the eigenvalue. The eigenvalues of more than 1 are considered significant (Kim and Mueller, 1987) to measure a new group of variables, namely Varimax Factor (VFs). The VFs coefficients that recorded a correlation of greater than 0.75 are considered as ‘strong’, 0.75 to 0.50 as ‘moderate’ and 0.50 to 0.30 as ‘weak’ significant factor (Liu et al., 2006). However, only factor loadings above 0.6 were taken into account for this study. 20 parameter variables will undergo PCA to determine the source of pollutants before hotspot analysis for the dominant source of pollutants in Malacca River.

Spatial Pattern Clustering Through Hotspot Analysis

Hotspot analysis can be clustered (spatial clusters) or individual (spatial outliers). In this study, spatial cluster of pollution would be water quality with a high value of parameter surrounded by a high value of pollutant sources. Meanwhile, spatial outliers of pollution include water quality with a high value of parameter surrounded by a normal or low value of pollutants source. The concept of hotspot can be expressed as:

$$G(d) = \frac{\sum\sum w_{ij}(d) x_i x_j}{\sum\sum x_i x_j}, \quad i \neq j \qquad \text{(Eq.9)}$$

where x_i is the value at location *i*, x_j is the value at location *j* if *j* is within *d* of *i*, and w_{ij}(d) is the spatial weight based on the weighted distance (e.g. inverse distance) (Getis and Ord, 1992). The expected value of *G (d)* is:

$$E(G) = \frac{\sum\sum w_{ij}(d)}{n(n-1)} \qquad \text{(Eq.10)}$$

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where *E (G)* is typically a very small value when *n* is large. A high *G (d)* value suggest a clustering of high values, and a low *G (d)* value suggests a clustering of low values. *Z* scores are used to evaluate statistical significance for *G (d)*. In other words, high positive *Z* scores suggest the presence of a cluster of high values or a hotspot, while high negative of *Z* scores suggest the presence of a cluster of low value or a cold spot (*Figure 2*).

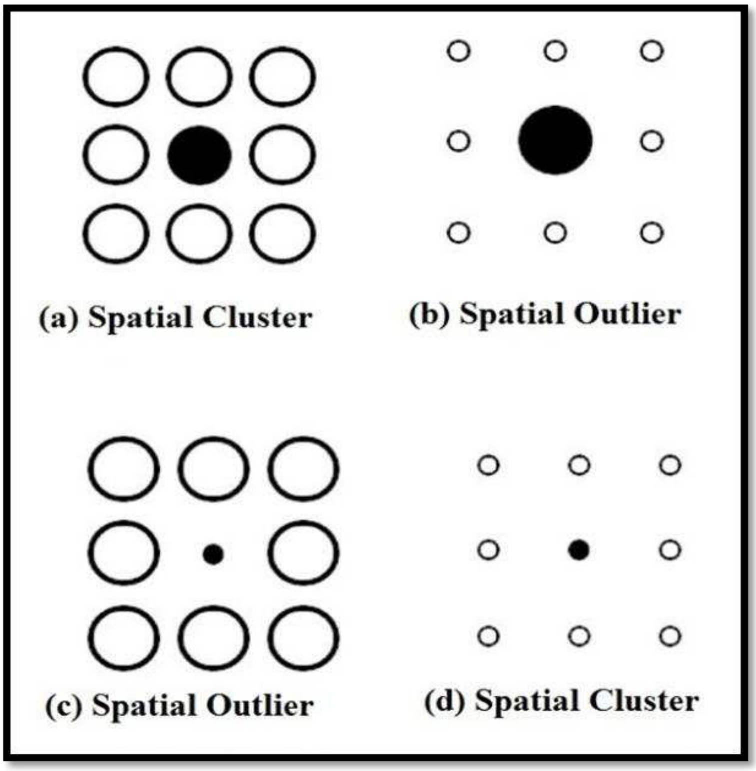

Figure 2. *The relationship of a location and its neighborhood; (a) and (b) are hotspot, (c) and (d) are cold spot*

Results and Discussion

Determination of Water Quality Status and WQI in the Malacca River

Water quality data of Malacca River (i.e. physico-chemical, biological, and trace element data) in comparison with the National Water Quality Standards (NWQS) (*Table 3* (i) and (ii) is as shown in *Table 4*. Based on *Table 4*, the result indicated that trace elements, together with pH and temperature, remained as class 1 in all sampling stations. Meanwhile, salinity in station 1 to 3 and station 7, together with turbidity in station 3, 8, and 9, was found to be in class 5. Only station 1 and 5 are in class 3 for turbidity, while other stations remained in class 2 and class 1 in terms of turbidity and salinity, respectively. Station 1 and 7 were found to be in class 5, station 2 and 3 to be in class 3, and station 4 to 6 were in class 1 for electrical conductivity and dissolved solids. However, only station 8 and 9 were in class 2 for electrical conductivity, while station 8 was within class 3 and station 9 was in class 1 for dissolved solids. For total suspended solids, most of the stations were classified as class 3, except for station 4, which was in class 4. BOD, COD, DO and NH_3N, in most of the water samples were classified as

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class 2 and class 3. However, station 2 and station 7 to 9 showed a class 4 BOD results. This includes NH_3N in station 1 to 3 and station 7 to 8 at class 4. Meanwhile, E. coli was classified as class 4 at station 3 and station 6 to 9, while the others were in class 5. Total coliform was found to be in class 5 for all sampling stations.

According to WQI (*Table 2*), majority of NH_3N and BOD parameters were in class 3, 4 or 5. Meanwhile, COD parameter from station 2 to 7 were in class 3, and others were in class 2. Both DO and TSS parameter showed that only several stations were in class 3. WQI trends showed that the water quality in Malacca River were declining from station 1 to station 7, which show that only station 4 and station 5 were polluted (class 4) while other stations were slightly polluted (class 3). Apart from that, station 8 and station 9 were still in clean condition, which is in class 2. Therefore, it can be said that all parameters value is affected (either decrease or increase) from the origins. These pollutants which came from human activities could cause problematical issues to the aquatic life in Malacca River.

Table 2. *WQI at sampling station in Malacca River sub-basin*

Water Quality Parameter	Unit	Water Sampling Station								
		S1	S2	S3	S4	S5	S6	S7	S8	S9
DO	mg/L	4.1	5.5	3.8	3.3	4.9	8.8	9.2	7.7	6.9
BOD	mg/L	5.0	6.0	2.0	4.0	4.0	7.0	6.0	5.0	2.0
COD	mg/L	22.0	77.0	61.0	53.0	47.0	38.0	29.0	13.0	21.0
AN	mg/L	1.4	0.8	2.6	4.8	7.2	3.3	0.7	1.8	2.1
TSS	mg/L	45	66	51	83	172	77	43	21	36
pH	-	6.5	6.3	6.4	6.4	6.8	6.5	6.6	6.3	5.8
Overall WQI		68	77	62	49	53	66	70	92	88
Class		III	III	III	IV	IV	III	III	II	II
Water Quality Status		Slightly Polluted	Slightly Polluted	Slightly Polluted	Polluted	Polluted	Slightly Polluted	Slightly Polluted	Clean	Clean

Table 3 (i). *National Water Quality Standards for Malaysia (adapted from Malaysian DOE, 2012)*

Category	Unit	Class					
		1	2A	2B	3	4	5
pH	-	6.5-8.5	6 – 9	6 – 9	5 – 9	5 – 9	-
Temp	°C	-	Normal+2°C	-	Normal+2°C	-	-
Sal	%	0.5	1	-	-	2	-
EC	µS/cm	1000	1000	-	-	6000	-
TSS	mg/L	25	50	50	150	300	300
DS	mg/L	500	1000	-	-	4000	-
Tur	NTU	5	50	50	-	-	-
BOD	mg/L	1	3	3	6	12	>12
COD	mg/L	10	25	25	50	100	>100
DO	mg/L	7	5 – 7	5 – 7	3 – 5	< 3	< 1
NH3N	mg/L	0.1	0.3	0.3	0.9	2.7	> 2.7
As	mg/L	-	0.05	0.05	0.4 (0.05)	0.1	-
Hg	mg/L	-	0.001	0.001	0.004(0.0001)	0.002	-
Cd	mg/L	-	0.01	0.01	0.01 (0.001)	0.01	-
Cr	mg/L	-	0.05	0.05	1.4 (0.05)	0.1	-
Pb	mg/L	-	0.05	0.05	0.02 (0.01)	5	-
Zn	mg/L	-	1	1	3.4	0.8	-
Fe	mg/L	-	1	1	1	1(leaf)5(others)	-
Total Coliform	count/100mL	100	5000	5000	5000(20000)	5000(20000)	> 50000
Ecoli	count/100mL	10	5000	5000	50000	50000	> 50000

Tur means Turbidity; DS means Dissolved Solid; Con means Electrical Conductivity; Sal means Salinity; Temp means Temperature; DO means Dissolved Oxygen; BOD means Biological Oxygen Demand; COD means Chemical Oxygen Demand; TSS means Total Suspended Solids; pH means Acidic or Basic water; NH_3N means Ammoniacal Nitrogen; E coli means *Escherichia* Coliform; Coli means Coliform; As means Arsenic; Hg means Mercury; Cd means Cadmium; Cr means Chromium; Pb means Lead; Zn means Zinc; Fe means Iron

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Table 3(ii). *Water Classes and Uses (adapted from Malaysian DOE, 2012)*

Class 1	Conservation of natural environment Water supply I – Practically no treatment necessary Fishery – Very sensitive aquatic species
Class 2A	Water supply II – Conventional treatment required Fishery II – Sensitive aquatic species
Class 2B	Recreational use with body contact
Class 3	Water supply III – Extensive treatment required Fishery III – Common of economic value and tolerant species; livestock drinking
Class 4	Irrigation
Class 5	None of the above

Identification of the Source of Pollutants

Based on the results of water quality status, it was found that parameters like E-coli, coliform, salinity, turbidity, NH_3N, and BOD were in the category of polluted conditions, while several parameters like EC, TSS, DS, COD, and DO were only slightly polluted. Other parameters remained as clean in Malacca River. Therefore, principal component analysis (PCA) was used to identify the source of pollutants that contributed to the pollution in Malacca River. As shown in *Table 5*, 7 PCs were obtained with eigenvalues more than 1, with 65% of total variance. The PC1 loadings with 14.7% of total variance have strong positive loadings on DS, EC, salinity and NH_3N. Physico-chemical parameters like DS, EC and salinity may be related to the erosion of riverbank due to dredging activities in the river and agricultural runoff from non-point source pollution (Juahir et al., 2011). The results were coupled with the NH_3N pollution together with salinity, highlighting the usage of pesticide for agricultural activities within oil palm and rubber plantations, and animal husbandry (chicken, cow, and goat) carried out within Malacca River basins. These activities contributed to the non-point sources of pollution that occured through surface runoff and water flows through the sub-basins before entering Malacca River. Additionally, PC2 loadings indicated a strong positive in terms of turbidity and TSS with total variance of 9.7%, which can be relate to interruption of human activities towards hydrologic modifications (such as dredging, water diversion, and channelization) that caused disruption in Malacca River (Daneshmand et al., 2011). Other activities like discharge from urban developments through land clearing (USGS, 2010) and surface runoff leading to erosion of road edges (Iscen et al., 2008) could also bring a small amount of pollution to the river.

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Table 4. *Mean (and standard deviation) values of water quality data along the Malacca River from in year 2015 (n=20)*

Category	Unit		S1	S2	S3	S4	S5	S6	S7	S8	S9
pH	-	Mean	6.50	6.43	6.46	6.46	6.55	6.49	6.64	6.40	6.33
		SD	0.34	0.28	0.31	0.43	0.21	0.31	0.35	0.34	0.47
Temp	°C	Mean	27.19	26.89	26.88	26.63	26.59	26.86	27.62	27.49	28.33
		SD	0.87	1.13	0.81	0.97	0.72	1.01	0.83	1.06	0.72
Sal	%	Mean	21.04	9.39	4.00	0.51	0.07	0.05	7.00	0.31	0.06
		SD	9.78	3.50	3.32	0.44	0.03	0.02	8.71	0.27	0.04
EC	µS/cm	Mean	16330.22	1403.73	1950.72	280.43	218.37	109.60	8173.90	1069.33	1093.83
		SD	12329.04	1067.37	1366.13	154.04	98.45	29.72	11118.65	459.17	630.46
TSS	mg/L	Mean	51.08	38.75	59.83	116.17	97.67	75.08	50.75	44.75	67.00
		SD	15.81	11.62	16.52	97.40	65.00	63.98	50.71	13.84	20.68
DS	mg/L	Mean	10444.61	1095.07	952.03	326.90	81.20	77.22	6257.26	505.89	223.00
		SD	7745.21	592.67	441.54	174.31	18.08	17.75	8716.84	214.39	136.15
Tur	NTU	Mean	116.70	73.61	584.57	99.86	121.18	84.85	63.62	297.77	209.71
		SD	67.18	29.59	494.93	70.09	65.26	30.01	47.17	128.61	276.70
BOD	mg/L	Mean	5.08	6.50	4.50	5.17	5.33	5.17	6.50	9.25	6.58
		SD	1.93	2.78	0.90	1.27	1.07	0.72	1.31	2.09	2.84
COD	mg/L	Mean	34.17	47.17	33.25	24.00	23.92	24.33	27.58	46.25	24.67
		SD	11.90	27.43	11.45	11.76	7.25	5.30	8.71	16.97	6.62
DO	mg/L	Mean	4.11	4.16	3.89	5.02	5.61	5.12	3.35	5.54	5.53
		SD	0.88	1.53	1.46	1.42	0.97	1.01	1.24	2.19	0.86
NH3N	mg/L	Mean	0.99	1.08	1.42	0.77	0.52	0.68	2.39	1.76	0.65
		SD	0.47	0.50	1.07	0.21	0.28	0.41	1.70	0.78	0.20
As	mg/L	Mean	0.0038	0.0040	0.0022	0.0026	0.0015	0.0022	0.0028	0.0037	0.0038
		SD	0.00426	0.00529	0.00147	0.00144	0.00080	0.00134	0.00119	0.00098	0.00204
Hg	mg/L	Mean	0.0002	0.0002	0.0002	0.0002	0.0002	0.0002	0.0003	0.0002	0.0002
		SD	0.00003	0.00009	0.00003	0.00000	0.00012	0.00000	0.00018	0.00000	0.00003
Cd	mg/L	Mean	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010
		SD	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Cr	mg/L	Mean	0.0016	0.0023	0.0017	0.0012	0.0011	0.0011	0.0011	0.0033	0.0011
		SD	0.00151	0.00372	0.00161	0.00035	0.00029	0.00029	0.00029	0.00444	0.00029
Pb	mg/L	Mean	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100
		SD	0.00000	0.00000	0.00000	0.00035	0.00000	0.00000	0.00000	0.00000	0.00000
Zn	mg/L	Mean	0.06	0.05	0.06	0.05	0.05	0.05	0.04	0.05	0.06
		SD	0.02	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02
Fe	mg/L	Mean	0.48	0.40	0.56	1.15	0.88	1.14	0.36	0.79	0.89
		SD	0.41	0.36	0.37	0.81	0.44	1.03	0.28	0.60	0.13
Total Coliform	count/100mL	Mean	698366.67	584866.67	701375.00	1005583.33	571808.33	656116.67	155958.33	117783.33	161558.33
		SD	510510.49	198044.61	448855.58	865227.40	340474.95	244295.76	107181.10	74326.53	94999.84
Ecoli	count/100mL	Mean	71275.00	63275.00	40358.33	17317.33	571808.33	20766.67	13745.00	14829.17	11455.83
		SD	33664.63	32261.69	43663.93	22922.74	340474.95	35458.43	6812.92	6701.57	7260.65

Tur means Turbidity; DS means Dissolved Solid; Con means Electrical Conductivity; Sal means Salinity; Temp means Temperature; DO means Dissolved Oxygen; BOD means Biological Oxygen Demand; COD means Chemical Oxygen Demand; TSS means Total Suspended Solids; pH means Acidic or Basic water; NH_3N means Ammoniacal Nitrogen; E coli means Escherichia Coliform; Coli means Coliform; As means Arsenic; Hg means Mercury; Cd means Cadmium; Cr means Chromium; Pb means Lead; Zn means Zinc; Fe means Iron; SD means Standard Deviation; S1 to S9 means Station 1 to Station 9

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On the other hand, PC3 loadings explained BOD loadings and COD loadings as moderately positive with 9.4% of total variance, which might be connected to anthropogenic activities and point source pollution like sewage treatment plants and industrial effluents (Juahir et al., 2011). Meanwhile, PC4 explaining E-coli loadings, coliform loadings, and pH loadings showed a moderate positive with 8.9% of total variance. The presence of E-coli and coliform indicated that animal husbandry, sewage treatment plant, and municipal wastes contributed to point source pollution in the river. This situation has caused the river water to absorb a large amount of oxygen and hence decreases the availability of DO, which indirectly underwent the anaerobic fermentation processes to produce ammonia and organic acid (Juahir et al., 2011). Consequently, acidic materials through hydrolysis have caused the water pH to decrease drastically. Next, PC5 explained moderate positive loadings on Zn and Fe with 8.4% of total variance. The Zn pollution can be linked to the large number of houses and building in the area that uses metallic roofs coated with Zn, which can mobilize into the atmosphere and waterways during acid rain or smog (Juahir et al., 2011), while Fe pollution can be attributed to metal group originating from industrial effluents (Aris et al., 2013). PC6 and PC7 loadings showed moderately positive Cr and Hg loadings having total variance of 7.4% and 6.4% respectively. Cr pollution can be linked to urban storm runoff (Juahir et al., 2011), and Hg pollution were suspected to link with plastic and chemical industrial wastewater (Papaioannou et al., 2010). Therefore, based on PCA analysis, 5 categories of pollutant sources were identified, namely agricultural activities, municipal and commercial residential activities, industrial activities, animal husbandry activities, as well as sewage treatment plant.

Classification of dominant pollutant sources

GIS Hotspot analysis was used to determine the dominant pollutant sources, which have been identified from PCA, namely agricultural, residential, industrial, and animal husbandry activities, as well as a sewage treatment plant, as shown in *Figure 3*. As described previously, *Z* score was used to evaluate the statistical significance for the variable in hotspot analysis. High positive *Z* scores suggest the presence of a cluster of high values or hotspots, while high negative *Z* scores suggest the presence of a cluster of low value or cold spot. For agricultural activity, the variable produced a general G-statistic of 0.0 and a Z score of 37.31, suggesting a spatial clustering of high value of 0.01 significant levels. Secondly, the residential variable has general G-statistic of 0.0 and a Z score of 74.72, with spatial clustering at a high value of 0.01 significant levels. Industrial variable indicated a general G-statistic of 0.0 and a Z score of 13.5 and suggested spatial clustering of high value of 0.01 significant levels., Animal husbandry activity showed general G-statistic of 0.0 with a Z score of -1.08, suggesting a spatial clustering of low values towards 0.10 significant levels. On the other hand, sewage treatment plant showed no value in general G-statistic and Z score, indicated that there are no significant level at the random value. Lastly, open space variable recorded a general G-statistic of 0.0 with a Z score of 28.73 indicating a high value of spatial clustering of 0.01 significant levels. All Z scores of selected variables were incorporated into GIS mapping to determine the dominant pollutant sources through hotspot analysis (*Figure 3*).

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Table 5. *Varimax rotation PCs for water quality in Malacca River basin*

Variables (Unit)	**Principle Components**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Turbidity (NTU)	-.104	.868	.016	.060	-.014	-.003	-.045
Dissolved Solid (mg/L)	.809	.027	.014	.076	-.128	.156	-.057
Electrical Conductivity (uS)	.859	.028	.003	.142	-.045	-.025	-.014
Salinity (ppt)	.808	-.075	-.212	-.124	.252	-.005	-.097
Temperature (°C)	.167	.171	-.204	-.384	.027	-.307	-.053
Dissolved Oxygen (mg/L)	-.367	-.283	.305	.415	.203	-.218	-.346
Biological Oxygen Demand (mg/L)	-.009	.049	.629	.095	.466	.334	.010
Chemical Oxygen Demand (mg/L)	-.056	.199	.609	.048	-.087	.022	-.070
Total Suspended Solid (mg/L)	-.193	.826	-.069	-.092	-.194	-.013	-.185
Acidity/Alkalinity (pH)	.081	.217	-.087	.745	-.201	.008	-.169
Ammociacal Nitrogen (mg/L)	.831	0.32	.011	.157	.128	-.010	.116
E-coli (cfu/100ml)	.533	-.048	-.124	.652	.359	-.108	.124
Coliform (cfu/100ml)	.027	.032	.293	.603	.096	-.192	-.221
Arsenic (mg/L)	.243	-.174	-.263	.011	.392	-.072	.047
Mercury (mg/L)	-.068	-.155	-.063	-.050	-.022	.065	.787
Chromium (mg/L)	.030	-.174	.037	.086	.322	.662	.267
Zinc (mg/L)	.146	.314	.014	-.091	.712	-.066	.156
Iron (mg/L)	-.114	-.258	-.051	-.068	.658	.028	.043
Initial Eigenvalue	2.650	1.751	1.692	1.609	1.517	1.336	1.157
% of Variance	14.723	9.729	9.398	8.939	8.427	7.422	6.430
Cumulative %	14.723	24.452	33.850	42.788	51.215	58.637	65.066

*The bold values are factor loadings above 0.60 that were taken after Varimax rotation are performed

Figure 3 (a) indicated the agricultural activities weighted concentration that concentrated in Kampung Tualang sub-basin (S5), which can be described as a hot spot area. The existence of a hot spot area in S5 sub-basin is due to the ease of access to water resources from Durian Tunggal Reservoirs. Indirectly, pesticide and chemical substances used for agricultural activities would enter surface runoff during the wet season. The water would flow into a nearby sub-basin before entering Malacca River. These processes contributed to the non-point source pollution. As shown in *Figure 3 (b)* residential activities shows that the high values are concentrated in Kampung Kelemak sub-basin (S1), Kampung Sungai Petai sub-basin (S2), Kampung Cheng sub-basin (S6), Kampung Batu Berendam sub-basin (S7), and a little bit at Kampung Tualang sub-basin (S5). There is also a highly weighted concentration located parallel to the Malacca River from S1 to S5. Only moderate values are shown along S6 to S9. Hence, residential activities showed that almost every sub basin is a hot spot area and hence it is a

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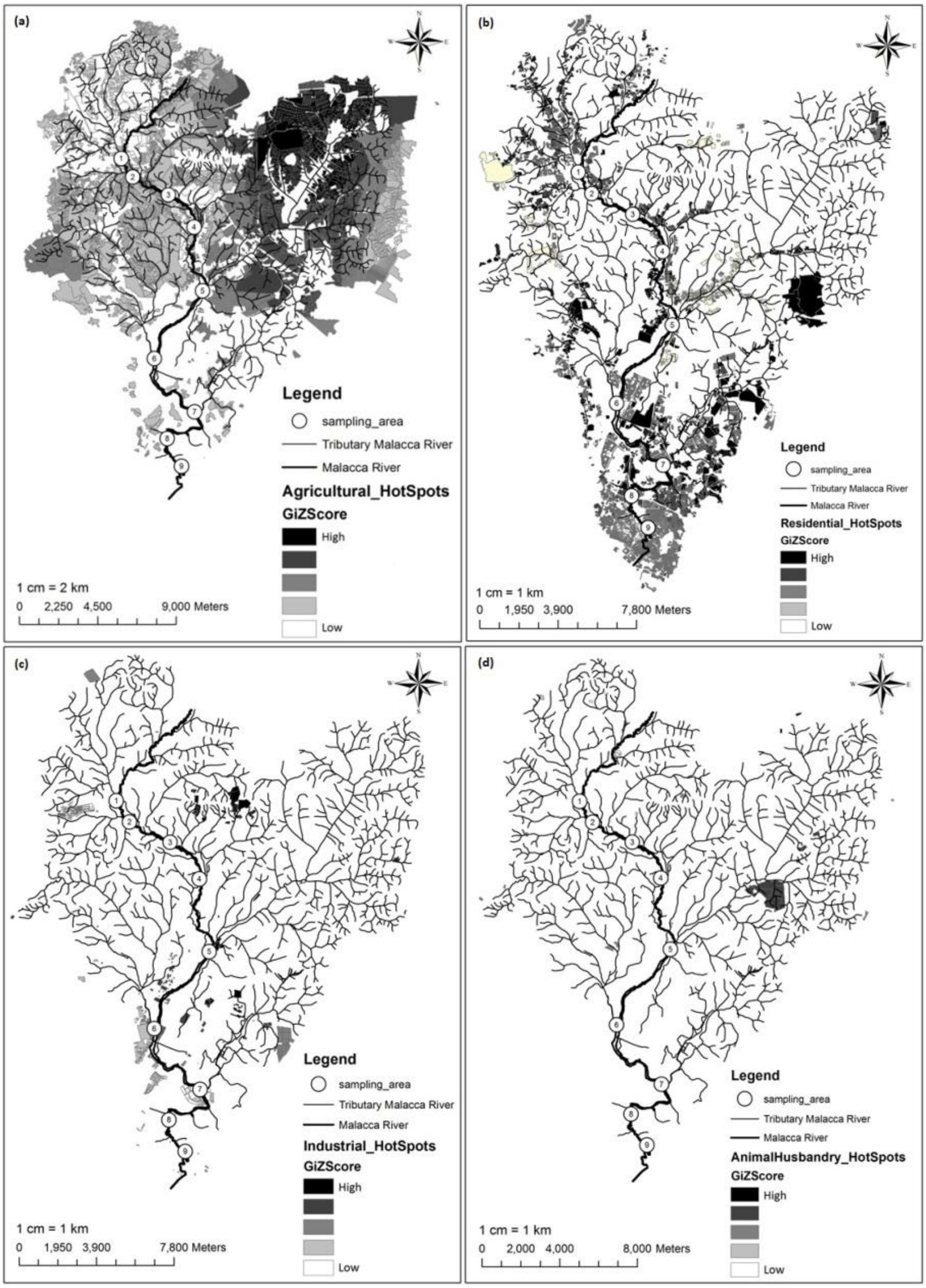
significant to contributor of pollution to the river. This situation may be related to the rapid and uncontrolled development, drastically increasing population, and unmanageable land clearing that brought pollution through wash water and cooking waste, municipal waste, and commercial waste as well as metallic roof pollution.



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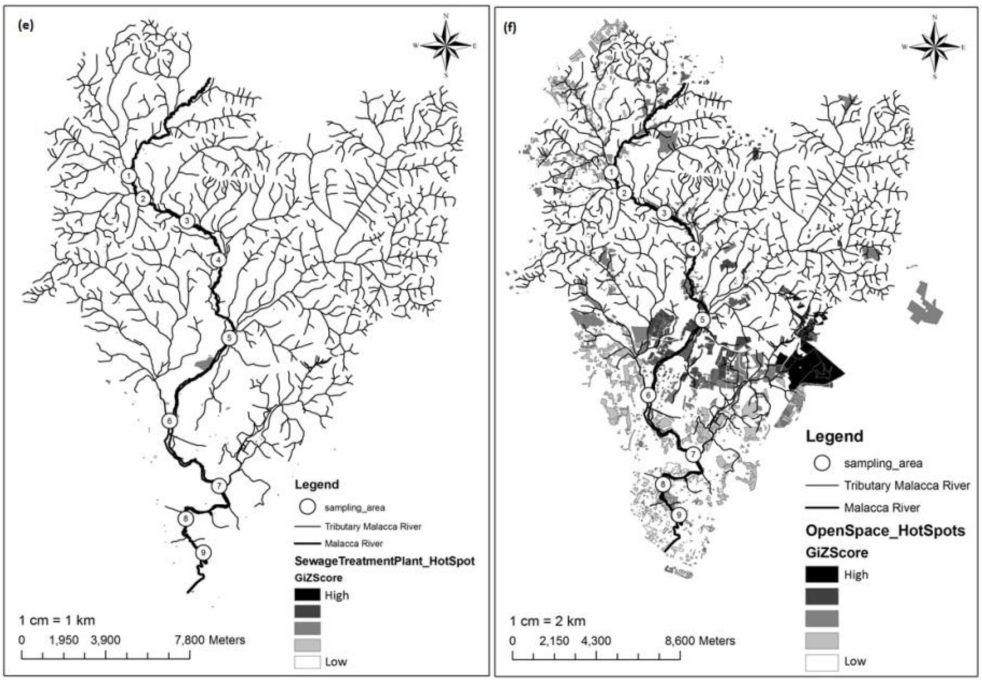

Figure 3. *Hotspot analysis of Pollutant Sources – (a) Agriculture; (b) Residential; (c) Industrial; (d) Animal Husbandry; (e) Sewage Treatment Plant; (f) Open Space*

Next, hot spot areas from industrial activities (*Figure 3 (c))* have been detected in Kampung Panchor sub-basin (S3), Harmoni Belimbing Dalam sub-basin (S4), Kampung Tualang sub-basin (S5), and Kampung Batu Berendam sub-basin (S7). High-technology and estate industries are the main contributors to point source pollution due to the direct discharge into sub basins before flowing into the main river. It is compulsory for industrial wastes to undergo treatment before being release onto surface water or in a river; however, certain industries refused to do so in order to save cost and time. Hence, these action increases the potential of hot spot area to pollute Malacca River sub-basins. Animal husbandry activities (refer to *Figure 3 (d))* shows a moderate hot spot area at Kampung Tualang sub-basin (S5) and several hot spots are scattered in sampling 1 to sampling 4 sub-basin, while the sewage treatment plant has a moderate hot spot area between S5 and S6 while others are scattered in S1 sub-basin and S6 to S9 sub-basins, respectively. Since animal husbandries are highlighted within a S5 sub-basin, this condition demonstrates that the activity is carried out in the area adjacent to Durian Tunggal Reservoirs as it is easier to obtain freshwater to feed the animals. However, unmanageable cleanliness within the farms led to animal feces flowing into the river through surface run-off, which contributed to the non-point source pollution. Sewage treatment plants that are scattered in downstream area can be clarified as low impact in terms of pollution in Malacca River, but they have a high chance to cause pollution if there is a malfunction that may lead to leakage (*Figure 3(e)).*

The open space variable shows a high value at Kampung Tualang sub-basin (S5) to act as a hot spot area, while moderate values were detected in S1 sub-basin and S6 to S8 sub-basins as shown in *Figure 3 (f).* Several moderate hot spot areas also exist along Malacca River, from sampling 1 to sampling 6. The main reason to have the open space variable in this study is to reduce river water pollution by controlling the pollutant source. This suggestion may be proposed to government sector agencies such as the

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Department of Environmental (DOE), Department of Irrigation and Drainage (JPS) and other departments that concerns with river water quality to build a monitoring system so that easi and frequent monitor of the water status could be done. At the same time, researchers and academicians may take the opportunity to develop studies on river water quality perspectives for a better environment.

Conclusion

This study has proven that PCA and GIS are remarkable and useful tools to discover the influential factors involved in Malacca River water quality. This study also revealed that sampling station 5 located in Kampung Tualang sub basin is considered to be the main area to cause pollution to the river through the dominant sources of pollutant from the agricultural, residential, industrial activities, and animal husbandry. Continuous exposure to pollutant sources concentration could pose a serious threat to the river's ecosystem in the present and future timeframe. Frequent assessment and monitoring is crucial for the continuous protection of Malacca River ecosystem. Therefore, this study does not only suggest the reduction of river water pollution by means of controlling the pollutant sources, but also by providing information which identifies the problematic areas for better management and understanding of the river water quality in the future. The study also provides a spatial database through GIS mapping for future reference for the development of proper land use and urban design procedures.

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DROUGHT PREPAREDNESS STATUS OF FARMERS IN THE NGUNI CATTLE DEVELOPMENT PROJECT AND THE SIRE SUBSIDY SCHEME IN NORTH WEST PROVINCE, SOUTH AFRICA

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Abstract. The objective of this study was to assess the drought preparedness status of the farmers that benefited from the Nguni Cattle Development Project and the Sire Subsidy Scheme in North West Province, South Africa. The data were collected using both quantitative and qualitative approaches to research, through a semi-structured questionnaire with five sections that probed into: A) personal information, B) farmer socio-economic resources, C) farm operational resources, D) access to Early Warning Information, and E) constraints to drought preparedness. Collected data were coded, captured and analysed using Microsoft Excel and SPSS (version 23) into frequency counts, percentages, graphs and tables. The results revealed that, majority of the respondents were African (97.6%); male (74.1%); married (64.3%); between 50 and 64 years of age (43.5%) and (38.8%) had high school education, respectively. Furthermore, most (76%) respondent farmers were not drought prepared with regard to grazing availability; 20% of the farmers were found to have non-dependable sources of water; 60% of the farms did not have any structural units for maintaining fodder banks and 63% did not have enough fodder reserves. Most respondents (84%) had never received drought related early warning information. Respondents cited lack of money (62%) and deficiency of information (58%) as the major constraints to their drought preparedness. The study recommends that the agricultural extensionists' plausible contact (95%) with the farmers should be used to also educate farmers on effective drought mitigation strategies and for dissemination of early warning information.
Keywords: *climatic changes, drought mitigation, early warning information, fodder banks, spare grazing*

Introduction

South Africa has recently experienced one of the worst droughts of the past century, with compromised agricultural production anticipated in the summer rainfall areas. The impact of droughts already severed the South African agricultural sector, largely affecting the extensive farming areas. In South Africa, drought has already cost farmers' losses of up to ten million rand (R10m) in 2015 (Bahta et al., 2016). Drought is however a common phenomenon in semi-arid and arid ecosystems (Coppock, 2011). Drought is furthermore the most frequent (Backeberg and Viljoen, 2003) and devastating phenomena that occurs in South Africa (Boone et al., 2004; Austin, 2008). Agricultural drought often has direct effects on the economy through the associations of agriculture with other sectors of the economy. This makes drought an important hazard, particularly in terms of the numbers of people affected (Coppock, 2011; Ngaka, 2012).

The uncertainty on the occurrence and magnitude of the effects of climatic changes, including the occurrence of droughts, often makes mitigation efforts quite problematic. Knowledge of the drought preparedness or vulnerability of the beneficiary farmers can assist the sector in proactive planning for drought mitigation and therefore reduce the pool of those who need relief in the event of droughts.

The North West Provincial Department of Agriculture has through its Sire Subsidy Scheme (SSS) and the North West/Industrial Development Corporation - Nguni Cattle Development Project (NW/IDC-NCDP) distributed genetic breeding material (beef cattle) to identified farmers throughout the province for over 10 years. The primary aim of the SSS is to induce herd genetic change in an attempt to improve the overall herd performance by the introduction of quality performance tested sires. The NW/IDC-NCDP on the other hand, is a tripartite partnership between the Department of Agriculture, the Industrial Development Corporation (IDC) and North West University (Mafikeng Campus) (DACE, 2008, Cwaile et al., 2012). The NW/IDC-NCDP was founded in 2006 (IDC, 2007) with the main aim of reintroducing and preserving the indigenous Nguni cattle genetic pool in the province (DACE, 2008).

All of the distributed genetic material is farmed under harsh extensive conditions, notwithstanding that, extensive beef production in the semi-arid and arid regions is highly vulnerable to climatic changes. On the other hand, North West Province has, as recent as in the years 2012 to 2015, been reported to have had the most devastating drought in 80 years. This is not surprising, considering that the year 2015 has been recorded as the driest year ever in South Africa since the recording of rainfall started in 1904 (SAWS, 2016). It is necessary therefore to collect all relevant data that could aid in assessing as to whether conditions under which the NW/IDC-NCDP and SSS cattle are farmed with, would allow for sustained production even in the event of the imminent droughts.

Drought preparedness involves vulnerability or resilience assessments, monitoring, forecasting as well as mitigation and response planning measures (Wilhite et al., 2005; Gutiérrez et al*.,* 2014). The main objective of the study was therefore to:

a) Determine the drought preparedness status of the farmers that benefited from the NW/IDC-NCDP and SSS in the North West Province.

The specific objectives of the study were to:

a) Determine the resource drought preparedness of the beneficiary farms.
b) Identify possible factors influencing drought preparedness of farmers.

Materials and Methods

The study was conducted in North West Province which occupies a total area of 104 882 km^2, covering 8.7% of the total area of the Republic of South Africa. The province is divided into four district municipalities, namely: Dr Ruth Segomotsi Mompati in the west; Dr Kenneth Kaunda in the south; Bojanala Platinum in the east, as well as the centrally located Ngaka Modiri Molema. North West Province borders the provinces of Northern Cape in the west, Free State in the south, Gauteng in the east and Limpopo in the north-east with the Republic of Botswana in the northern side. Much of the province consists of flat areas of scattered trees and grasslands. The rainfall pattern of the

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province is highly variable, both spatially and temporally. Under normal climatic conditions, the western parts of the province receive on average less than 300mm per annum, the central parts around 550mm and the eastern and south-eastern parts receives over 600mm per annum.

The data were collected using both quantitative and qualitative approaches to research. This was done in an effort to improve the validity, analytic power and relevance of the findings. The qualitative approach documented the respondents' perceptions and opinions through in-depth interviews while the quantitative approach was used to capture the *in-situ* numerical and physical observations on infrastructure. Data were collected through a semi-structured questionnaire made up of five sections, namely: A) personal information, B) farmer socio-economic resources, C) farm operational resources, D) access to Early Warning Information (EWI), and E) constraints to drought preparedness. Face to face interviews as well as physical observations, and assessment of the farms' resource base were used to collect the data. An inclusive total of 85 beneficiaries from all four districts of North West Province were interviewed in this study. The collected data were coded, captured and analysed using Microsoft Excel (2010) and SPSS (version 23) into frequency counts, percentages, graphs and tables.

The SSS had not been rolled out in all the districts and therefore, proportional representation of the SSS against the NW/IDC-NCDP could not be achieved (*Fig. 1*). Only beneficiaries who were actively involved in the project were targeted for the NW/IDC-NCDP. This was because, once the beneficiaries have returned their loaned cattle, there is essentially no contractual obligation on their part to partake in departmental programmes.

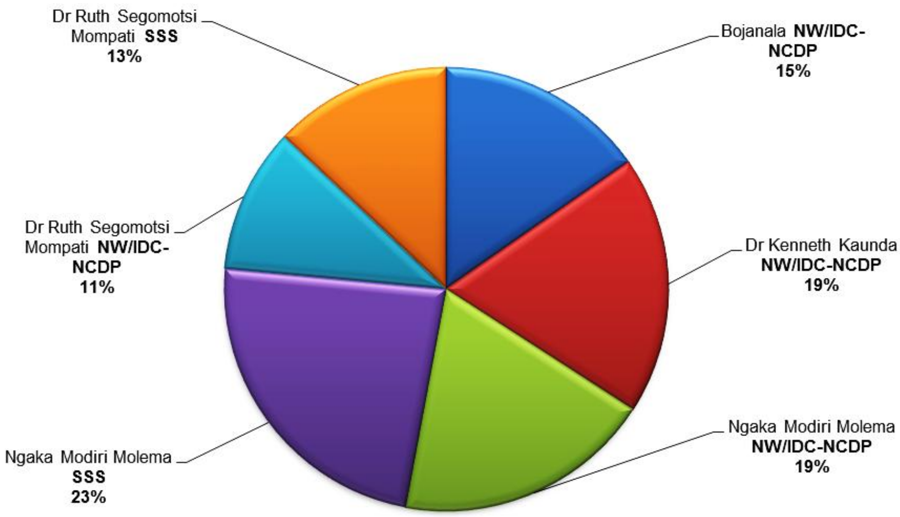

Figure 1. *Data origin and distribution per district*

Results

Socio-economic and demographic characteristics of the respondents

The distribution of respondents according to various socio-economic and demographic characteristics is summarised in *Table 1*. The results revealed that most of

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the respondents were African (97.6%), male (74.1%), married (64.3%), between 50 and 64 years of age (43.5%) and (38.8%) had high school education, respectively. For the purposes of this report, high school education refers to educational programmes consisting most commonly of students/pupils enrolled in grades 10 through to 12. A noticeable number of the respondents (31.8%) had tertiary education. Most respondents (49.4%) had a substantial household size of about 5 to 8 members. This is so despite most respondents (51.2%) having on average, 1 to 3 dependants. The results further show that young farmers (aged 34 years and less) accounted for only 9.4% of the respondents. More than half (51.8%) of the respondent farmers never received any training or short courses in agriculture.

Table 1. *Socio-demographic characteristics of respondents*

Characteristic	Sub-characteristic	Frequency	Percentage
Gender	Female	18	21.2
	Male	63	74.1
	Group with both genders	4	4.7
Age	20-34	8	9.4
	35-49	20	23.5
	50-64	37	43.5
	65-79	18	21.2
	≥ 80	2	2.4
Marital status	Single	22	26.2
	Married	54	64.3
	Widowed	6	7.1
	Divorced	2	2.4
Race	African	83	97.6
	Coloured	1	1.2
	Indian	1	1.2
Dependants	0	8	9.5
	1-3	43	51.2
	4-6	27	32.1
	7-9	6	7.2
Household size	1-4	35	41.2
	5-8	42	49.4
	9-12	7	8.2
Educational	None	2	2.3
	Primary	13	15.3
	Secondary	10	11.8
	High School	33	38.8
	Tertiary	27	31.8
Agriculture short courses	Yes	41	48.2
	No	44	51.8

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Farmer socio-economic resources

The results revealed that 88% of the respondents were full-time farmers and only 8% of them were farming on a part time basis (*Table 2*). Groups that included both full time and part time farmers represented a very small part (4%) of the respondents. Furthermore, 73% of the respondent famers did not engage in non-farming business activities. The results further revealed that 76% of the respondents had no other income to help them during droughts.

Table2. *Summary of responses on farmer socio-economic resources*

Characteristic	Sub-characteristic	Frequency	Percentage
Type of farmer?	Full time	75	88
	Part time	7	8
	Both (as a group)	3	4
Do you engage in non-farming business activities?	Yes	23	27
	No	62	73
Do you have other income to help you during droughts?	Yes	20	24
	No	65	76

Farm operational resources

Responses and observations on farm operational resources in relation to available grazing are summarised in *Table 3*. On farm observations revealed that only 43 (51%) of the respondent farms seemed to have reasonable amounts of grazing for a season. Of the 43 farms, 31 (72%) had enough grazing to last for 5 to 6 months. The remaining farms had either enough grazing for 1 to 2 months (9%) or 3 to 4 months (19%). However, from the 43 farms with reasonable amounts of grazing, only 35 (41%) had spare or reserved grazing, of which only 57% of such spare grazing could sustain a herd of cattle for 5 to 6 months. In essence, out of the studied population (85), only 31 (36%) of the farms had enough grazing to last for 5 to 6 months, and only 20 (24%) of these farms had enough spare grazing to last for 5 to 6 months. These results indicate therefore that the remaining 65 (76%) respondent farms did not have enough grazing to sustain their cattle for two seasons of the year.

It is further revealed in *Table 3* that boreholes were the most prominent (86%) source of drinking water for cattle. The majority of the respondents rated their sources of water as either highly dependable (54%) or dependable (26%). The remaining respondents (20%) who rated their water source as non-dependable, had either a too small/non-functional reservoir or only one borehole (16%), whereas a minority (4%) had no source of water at all on the farm.

Table 3 also shows that 60% of the respondent farms did not have any structural units for maintaining fodder banks. Furthermore, 84% of the respondent farms did not have enough fodder reserves. The reasons advanced by farmers for not having stored fodder are also summarised in *Table 3*. Of the 84% respondents without enough stored fodder, only 21% had at least enough standing hay as back-up fodder for periods of shortages. The remaining 63% did not have enough fodder reserves. It is worth mentioning that among the reasons advanced for not maintaining fodder banks, the combined frequencies of “lack of knowledge” and “ignorance” accounts for 38% of the

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reasons why respondents did not have any fodder stored. Lack of funds accounted for a further 23%, whereas lack of infrastructure and equipment respectively accounted for 17 % and 1% of the reasons why respondents had no reserved fodder.

Table 3. *Summary of responses and observations on farm operational resources*

Characteristic	Sub-characteristic	Frequency	Percentage
Does the farm have enough grazing?	Yes	43	51
	No	42	49
If yes, for how long?	1-2 months	4	9
	3-4 months	8	19
	5-6 months	31	72
Does the farm have spare grazing for the herd?	Yes	35	41
	No	50	59
If yes, for how long?	1-2 months	5	14
	3-4 months	10	29
	5-6 months	20	57
Does the farm have adequate fodder banks?	Yes	34	40
	No	51	60
Does the farm have enough fodder in fodder banks?	Yes	13	15
	No	71	84
What is your source of water for cattle	Borehole	73	86
How dependable is your water source	Dependable to Highly dependable	68	80
	Not dependable	17	20
Reasons for not having stored fodder	No infrastructure	12	17
	No funds	17	23
	Lack of knowledge	12	17
	Ignorance (Do not know)	15	21
	Have enough standing hay (grazing)	15	21
	Lack of equipment	1	1

Source of extension messages

Figure 2 shows that 95% of the respondents had contact with agricultural extensionists and that 64% of such contact was on a regular basis. The main source of extension messages (94%) with regard to drought were reported to be from government extension services (*Fig. 3*). This can be expected given that 65% of the respondents did not belong to any farmer association or organised agriculture groupings. A negligible number (6%) of respondents received extension messages from non-governmental sources as well.

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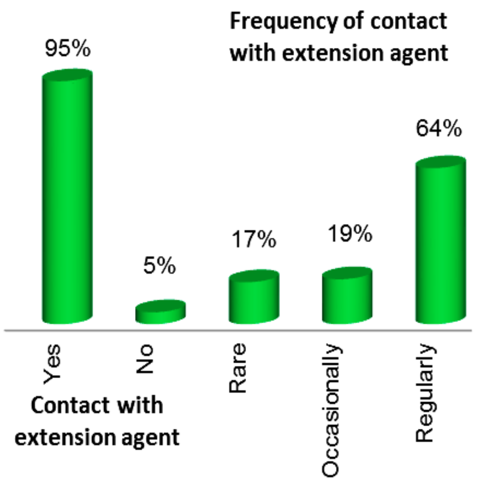

Figure 2. Respondents' contact with extension agents

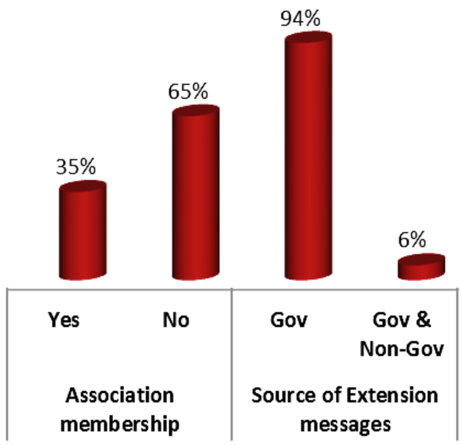

Figure 3. Membership of associations and source of extension messages

Early warning information

Access to early warning information and drought planning among the respondent farmers is summarised in *Table 4*. The results indicate that 98% of the respondents reported that they earnestly need EWI, and 91% of them expect such information to come from the state extension services.

Table 4. *Access to early warning information and drought planning*

Characteristic	Sub-characteristic	Frequency	Percentage
Have you directly received any advice on what to do before and during drought?	Yes	15	18
	No	70	82
Have you directly received any EWI about drought?	Yes	14	16
	No	71	84
Do you need EWI for drought preparation?	Yes	83	98
	No	2	2
From where can you get expert advice/information about drought?	Extension Officers (EO)	77	91
	EO and SA Weather services	3	3
	I do not know	4	5
	Climatologists	1	1
Do you have an existing drought plan?	Yes	37	44
	No	48	56
Reasons for not having a drought plan?	Lack of knowledge	40	73
	The farm has enough standing hay	8	14
	I usually buy fodder as needed	3	6
	Combination of both the above	4	7

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However, 84% of the farmers have never directly received EWI with reference to neither their specific farming area nor their farms. Furthermore, 82% of the respondents reported that they have never directly received advice on what to do before or during drought for purposes of enhancing their drought preparedness. More than half (56%) of the farmers did not have any drought mitigation plan at all. *Table 4* further revealed that 73% of the respondents cited lack of knowledge as the major reason for not having any drought plan.

Land utilisation

Proportional land use subdivisions among respondent farms are summarised in *Table 5*. The individual farm sizes vary considerably within and across district municipalities and range from 4 hectares in Ngaka Modiri Molema to 3600 hectares in Dr Ruth Segomotsi Mompati. The average farm size for the studied population was found to be 709.51 ha with a standard deviation equalling 507.94. Most of the respondents are farming extensively on veld (89%) with very little (4%) planted pastures (*Table 5*). The available arable land (7%) is often cultivated and of reasonable use to livestock for drought preparedness purposes.

Table 5. *Farm land (ha) size statistics and proportional subdivision*

Item *N* **(71)**	**Comparative land subdivision**			
	Portion veld	**Portion pasture**	**Portion arable**	**Total land size**
Mean	635.01	25.68	47.67	709.51
S.D.	517.61	83.18	89.12	507.94
Min	2	10	2	4
Max	3600	430	388	3600
Sum	45094	1849	3432	50375
Proportional use	89%	4%	7%	100%

Farm size and livestock statistics

Farm size and livestock statistics per district are summarized in *Table 6*. On average, the largest farms were observed in Dr Ruth Segomotsi Mompati, and respectively followed by the Dr Kenneth Kaunda; Bojanala Platinum and Ngaka Modiri Molema districts. The largest herds (mean) were surprisingly observed from the Dr Kenneth Kaunda district (113) followed by Bojanala platinum district (71). The herds with the least number of cattle (2 and 17) were observed from the Dr Ruth Segomotsi Mompati and Ngaka Modiri Molema districts respectively. The results with regard to farm and herd sizes are not surprising, and are largely in accordance with the grazing potential (natural veld) of the specific areas (*Fig. 5*) in terms of hectares per large stock unit (ha/LSU). For instance, the smallest herd (2 cattle) was observed from a communal area in Taung with a grazing potential of 20-25 ha/LSU, followed by herds from the Mafikeng area with a grazing potential of 18 ha/LSU (*Fig. 5*).

Grazing in Dr Ruth Segomotsi Mompati was mainly made up of natural veld (100%), followed by the Bojanala Platinum district (96%) and Dr Kenneth Kaunda district (84%) as well as Ngaka Modiri Molema (79%). In addition to natural grazing, the balance of land utilization percentages in *Table 6* represented arable land under dryland production as well as cultivated pastures.

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The majority of the farms are leased (61%) and only 14% of respondents own the land that they are farming on (*Fig. 4*). This may be contributory to the observed frequency (17%) of "no infrastructure" regarding lack of fodder banks in *Table 3*, as farmers may be reluctant to make improvements on leased land. A further 17% and 8 % of the land was respectively utilized through communal and tribal allocation.

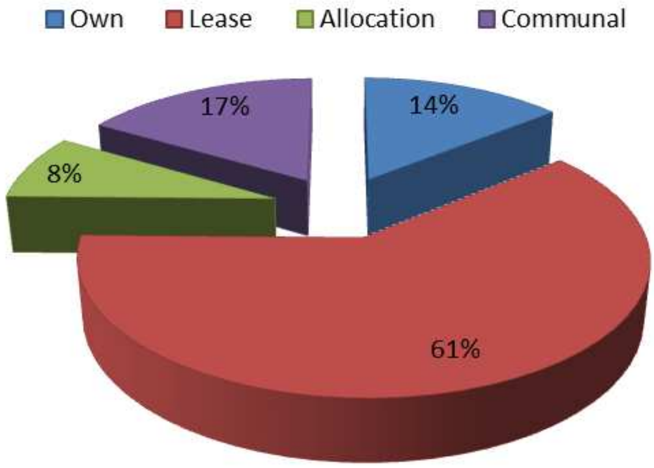

Figure 4. *Land ownership*

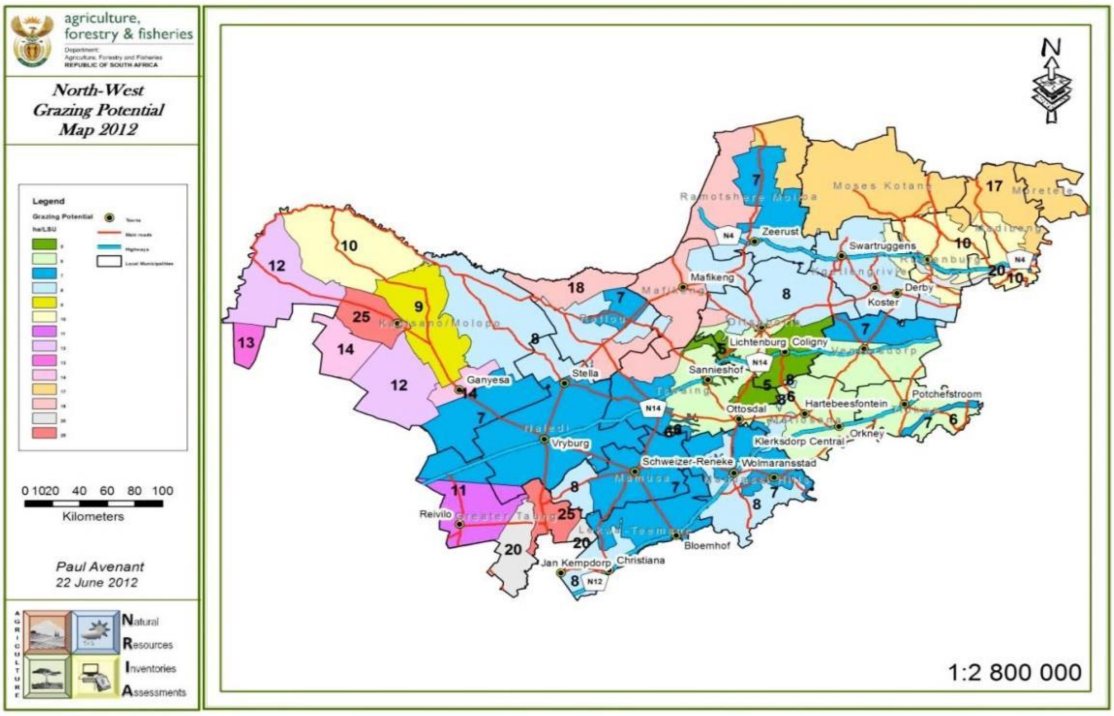

Figure 5. *Grazing potential map for North West Province, South Africa*

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Table 6. *Summarized farm size and cattle statistics per district (cattle in parenthesis)*

Item	Dr Ruth Segomotsi Mompati	Dr Kenneth Kaunda	Bojanala	Ngaka Modiri Molema
n	17	16	13	25
Land size (ha)				
Mean	978 (64)	723 (113)	592 (71)	579 (64)
Min	314 (2)	250 (35)	340 (33)	4 (17)
Max	3600 (110)	1777 (297)	1000 (110)	1600 (194)
Percentage Natural veld	100	84	96	79

Constraints to drought preparedness

Out of a number of possible constraints to drought preparedness, mainly three constraints were cited by all respondents (*Table 7*). It is revealed in *Table 7* that lack of government support was the least (42%) of the constraints to drought preparedness. However, lack of money (62%), followed by lack of information (58%) were cited as the two biggest constraints to drought preparedness.

Table 7. *The most cited constraints to drought preparedness*

Factor	Sub-factor	Frequency	Percentage
Lack of government support	Yes	36	42
	No	49	58
Lack of information	Yes	49	58
	No	36	42
Lack of money	Yes	53	62
	No	32	38

Discussion

Socio-demographic characteristics are of significant importance when studying drought preparedness or vulnerability of farmers. This is because the socio-demographic characteristics are likely to have varied influence on human behaviour in response to drought. For example, Raphael et al. (2009) found that the odds of perceived drought being very likely to continue, were significantly higher in women than in men when considering the concern for self or family. Similarly, some studies found that as a demographic characteristic, gender had a critical influence in decisions on drought and agricultural activities (Bahta et al., 2016; Muyambo et al., 2017). The results confirm that animal production is a male dominated career in the studied area, with very little youth involvement. In agreement, Cwaile et al. (2012) found the majority of the Nguni cattle beneficiaries to be males aged above 60 years, and Oladele et al. (2013) found that the youth accounted for only about (7%) of respondents in their study. The lower level of participation of younger persons in this study is suggestive of compromised youth empowerment with reference to the two schemes understudy. The lower participation of the youth may be further exacerbated by capital requirements for cattle

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farming, particularly access to land. Youth within close proximity of the surveyed demographic population did not own or have much access to land and therefore had limited chances of participating in cattle farming. In line with these socio-demographic characteristics, it is not surprising that the majority of respondent farmers were not drought prepared.

The finding on respondent education is in agreement with the national census (Stats SA, 2011), which declared that among the South African population, 35.2% of black/African citizens have completed an education level of high school or higher. However, this level of education did not seem to encourage drought preparedness in anyway.

The results on farmer socio-economic resources suggest that the majority of the respondents have ample opportunity to decisively focus on farming activities, including drought preparedness. The results further confirm that a great majority of the farmers are dependent on their farming enterprises for income. It is therefore important for these famers to be drought prepared since their entire livelihood is dependent on their farming enterprises.

Considering that all the farms are managed as extensive farming units, a conclusion can be drawn from the result on farm operational resources that the respondents were already prone to grazing shortages and therefore vulnerable to drought at the time of the study. These findings point to a worrisome scenario, particularly when considering that the frequency and impact of natural disasters like drought has significantly increased in recent years (Ngaka, 2012), with further increase in rainfall variability expected for Southern Africa (Van Riet, 2012). In addition, North West Province has as recent as 2015, been reported to have experienced the most devastating drought in about 80 years.

Fodder banks are concentrated units of stored forage, established for storing and providing additional feed for livestock during the dry season or periods of extended grazing shortages. The frequency of "lack of funds" as a reason for not having fodder banks is suggestive that the affected farming enterprises do not generate enough income to cater for drought preparedness activities.

There is general consensus that, farm adaptability is an important link between sustainable agriculture and drought vulnerability (Wall and Smit, 2005; Thomas et al., 2007; Bryan et al., 2009; Knutson et al., 2011). Efforts should therefore be made to enhance farm adaptability to climatic changes and consequently to drought. This is more so when considering that, it is often necessary for farmers to make certain climate influenced decisions several months before the impact of the climate variation is realized (Vogel et al., 2000; Du Plessis and Van der Waal, 2004).

Literature predictions suggest that, the effects of climate change on the southern hemisphere will present more frequent and pronounced periods of droughts (Bryan et al., 2009; Meissner et al., 2013; Scholtz et al., 2013). Therefore, although 80% of the respondents had dependable to highly dependable sources of water, South Africa is a water stressed country and a need to plan for periods of hydrological droughts and manage water availability as well as accessibility remains very important. The desirable availability of water in this study should be interpreted with caution, particularly because hydrological drought tends to lag behind meteorological drought with some temporal margin. For instance, Edossa et al. (2010; 2014) found that the lag time between the hydrological and meteorological drought events is often in the range of 4 – 12 months.

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The observed plausible contact with agricultural extensionists in this study is probably encouraged by the fact that, regular monitoring of the projects is an inherent job expectation on the part of the agricultural extensionists. It is however surprising that the levels of awareness of the respondents with regard to drought preparedness and EWI does not correspond to the reported magnitude of contact with agricultural extensionists. This result is suggestive of a discord with regard to the relevance of the extension messages delivered on the observed contact junctures. In support, Maphodi et al. (2014) postulated that lack of adequate and relevant updated knowledge on various subject areas by agricultural extension officers might be the stumbling block for useful agricultural extension services to be rendered.

The results suggest that there is a pronounced inadequacy with regard to dissemination of EWI to farmers particularly with regard to droughts. This may consequently explain in part why 56% of the respondents did not have any existing drought plan. This is of concern, particularly when considering that most of the respondents had regular contact with agricultural extension services. The inadequacy in the dissemination of EWI on drought in the study area is a direct challenge to both the departmental Disaster Risk Management (DRM) and the agricultural extension services. The responsibility of the dissemination of any agricultural drought related information is therefore squarely on government, particularly by agricultural extension services. This is more so when considering that the results further showed that 65% of respondents were not affiliated to any farmer's association and 94% of the received extension messages, were from government extension services.

A conundrum remains, if extensionists are having such plausible contact (95%) with the studied farmers, how does it happen that 84% of these farmers have never received EWI and were never instructed on drought mitigation strategies? While this question remains a subject of a different study, this puzzle is nonetheless further suggestive of a discord of responsibilities between extension services and DRM within the provincial department of agriculture. Additionally, these findings highlight in essence, the possible need to provide intensive extension workers' training on interpretation and conversion of EWI into extension messages.

The large standard deviation in land utilisation indicates the degree to which individual farms differ from the overall mean of all surveyed farms. This is suggestive of a large size variation between farms. As a result therefore, all assistance for drought preparedness will need to be tailor-made, since one strategy may not be applicable to all farms. Furthermore, both extensive beef and dryland production systems as depicted from this study are highly dependent on climatic conditions, consequently increasing the vulnerability prospects of the respondents to climatic variations, particularly drought. This is of great concern, particularly when considering the general consensus that southern Africa will become warmer and drier (Meissner et al., 2013) with overall increases in drought frequency (O'Farrell et al., 2009). These impacts can be expected to amplify the vulnerability of livestock production systems (Gill and Smith, 2008; Rust and Rust, 2013).

The results on herd size are contrary to the findings of Stats SA. (2011), where the largest herds in the country were observed in the Dr Ruth Segomotsi Mompati district. This contrast is probably as a result of the effects of the recent droughts, where areas with less rainfall had the most stock reductions.

It can be deduced from these results that more than half of the respondents are satisfied with government support in general but have significant challenges with

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funding and relevant knowledge/information on drought mitigation strategies as well as preparedness. Similarly, Maphodi et al. (2014) found that although 84.7% of farmers had access to government support, 84.2 % of them indicated that they had financial problems. The role of government regarding financial assistance to farmers, and that of extensionists in educating farmers on effective drought management strategies can as a result never be over emphasised.

Suggestions and conclusion

Suggestions for drought preparedness

The high frequency of drought vulnerability among the farmers, as well as the looming climate change and its associated effects suggests a need for proactive farm management practices and interventions, in preparation for drought eventualities. To ensure the realisation of drought preparedness of the surveyed farmers, the following interventions are suggested:

- Agricultural extensionists should acquire foreknowledge and information on anticipated climatic changes, for them to be able to timeously guide and assist vulnerable farmers with regard to mitigation and coping strategies.
- Famers need to appropriately manage their production systems to achieve high operational efficiency for income generation, in order for drought preparedness costs to be absorbed by their operations.
- The extensionists' plausible contact with famers should among other things, be used to educate farmers on proactive resource management for enhancing farm adaptability to climatic changes as well as on effective drought mitigation strategies for increasing farm resilience.
- The provincial department of agriculture should develop and enforce an obligatory institutional information exchange protocol between DRM and extension services for EWI.
- The provincial department of agriculture should prioritise the development and dissemination of EWI to administrative officials and farmers respectively.
- Subsequently, concerted efforts on the part of extension services should be made to repackage extension messages in such a way that they are spatially and temporally relevant to drought related needs of the farmers, and to create avenues on which regular area specific updates can be delivered to farmers.

Conclusion

This study revealed that the majority of farmers were not drought prepared and had very little knowledge regarding drought planning and management thereof. Grazing availability was exclusively a key determinant of drought vulnerability. Access to drinking water for cattle seemed to have less influence on the vulnerability of the surveyed respondents. A pronounced inadequacy with regard to dissemination of EWI to farmers particularly with regard to droughts was observed. Agricultural extensionists had a plausible contact with most farmers on a regular basis. Such contact was however not used to educate farmers on effective drought mitigation strategies.

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APPLICATION OF CA-MARKOV MODEL AND LAND USE/LAND COVER CHANGES IN MALACCA RIVER WATERSHED, MALAYSIA

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Abstract. Modeling the effects of past and current land use composition on surface water quality of the Malacca River watershed provides valuable information for environmental and land planning. The Land use land cover (LULC) spatio-temporal changes in watershed are classified as non-industrial area, industrial area, vegetation area, open space area, water bodies, as well as farming area. This is compared with the future spatial pattern simulated using CA-Markov model to evaluate qualitative and quantitative changes of LULC over time (between 2001, 2008, 2015, and 2029). This analysis consisted of the entire Malacca River watershed area. Changes may provide information on determinant of river water quality in future. Based on LULC monitoring and future simulation approach, the results may be used in an early warning system by demonstrating trend and consequences of changes in watershed. Continuous downward trends for vegetation area, open space area, and water bodies are due to expansion in non-industrial area, industrial area, and farming areas that increase contamination in the Malacca River. The findings demonstrate the need for more regulation in land use policy design, planning, and development for the protection of river water quality.
Keywords: *land use land cover changes, CA-Markov model, land-sat, quantitative and qualitative simulation*

Introduction

Anthropogenic activities negatively affect the conditions of the world's water. Various studies have estimated that half of the world's water quality has decreased due to human activities (Aris et al., 2013; Najar and Khan, 2012), and this condition has continuously worsened until today (Hua, 2017; Rosli et al., 2015). According to Hua et al. (2016), the main characteristic to cause contamination and detected as pollutant sources are residential, industrial, and agricultural activities. This matter is confidently supported by a majority of researchers (Aris et al., 2013; Hua, 2017; Hua et al., 2016; Rosli et al., 2015; Wan et al., 2014). Human alteration of cities engineered toward the land use land cover (LULC) change drastically results in negative impact on water quality, especially in rivers (Wan et al., 2014; Yang et al., 2012). Based on the evidence, contaminated rivers could threaten aquatic animal, reduce the availability of quality water. This will affect the flora and fauna, as well as affecting the climate at a regional scale (Carey and Fulweiler, 2012; Wan et al., 2014). Hence, it has become essential to obtain an understanding of how recent trajectories of land use will manifest in the future (Parsa et al., 2016; Subedi et al., 2013).

LULC change models are usually applied to detect location of the changes occurred or will potentially occur (Halmy et al., 2015; Yagoub and Al Bizreh, 2014). By determining the factors of the changes, the models benefit through providing probabilistic prediction of possible changes may occur (Halmy et al., 2015). Technically, change analysis is carried out

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using historical land use data in which the past land transformations and transitions were assessed. The transition trend is incorporated with environmental variables to provide an estimate of future scenarios (Behera et al., 2012; Yagoub and Al Bizreh, 2014). Prediction of LULC changes is important to understand and highlight the potential modifications and alterations that might be happen to the landscape in the near future. Typically, changes occur due to increasing population, distance to roads or other facilities, type of the soil, environmental issues, and so on. LULC models are usually used to assess the cumulative impact of land use changes and develop the future scenarios (Behera et al., 2012; Halmy et al., 2015), which is important in providing support and help to land use planners, resource managers, and conservation practitioners in making decisions (Halmy et al., 2015; Memarian, et al., 2013; Parsa et al., 2016). Simultaneously, prediction LULC changes model provide advantages in different applications, such as urban planning through modeling rural development and urban growth (Arsanjani et al., 2013); selecting conservation priority areas and setting alternative conservation measures (Subedi et al., 2013); studying dynamics of shifting cultivation (Halmy et al., 2015); as well as simulating the land use range dynamics under different watershed area (Behera et al., 2012; Memarian, et al., 2013).

Markov chain analysis works on the assumptions of physics in which the probability of a system being a certain state at certain time can be determined if the earlier time state is known (Arsanjani et al., 2013; Behera et al., 2012; Halmy et al., 2015). In other words, Markov chain analysis uses statistical spatially dependent land use data required by logistic regression (Arsanjani et al., 2013). This method is based on developing a transition probability matrix of land use change between two different dates derived from observation, which will be used to provide estimations of the probability that each pixel of certain LULC classes will be transformed to another class or remain in the same class (Yagoub and Al Bizreh, 2014). Nevertheless, Markov chain analysis has several issues as the methods only provide short-term projection (Sinha and Kumar, 2013) and are not spatially explicit (Halmy et al., 2015), as it does not provide spatial distribution of the changes that might occur to understand more about the potential impact of the projected changes. These issues can be overcome through suggestion of integrated modeling approaches with other different dynamic and empirical methods considered suitable for modeling land use changes processes (Halmy et al., 2015; Sinha and Kumar, 2013).

The Markov chain model can be integrated with the cellular automata model (or CA-Markov model), and these models have been widely used in different scale especially involve with modeling and predicting the land use change (Arsanjani et al., 2013; Subedi et al., 2013). The CA-Markov model is applied in this for the advantages of the combination of the stochastic aspatial Markov techniques with the stochastic spatial cellular automata method (Arsanjani et al., 2013). These include the prediction of two-way transitions among the availability of LULC classes rather than the Geomod technique that only concentrates on prediction of one way loss or gain in one classes (Halmy et al., 2015). Hence, transition-based models are the integration between aspatial Markov model with spatial cellular automata model to performed the regression-based models in predicting the land use change. This study has been carried out to provide information on the presence of LULC patterns in the Malacca River watershed and aims to analyze the changes of landscape trend pattern using the past LULC, the presented LULC, and future LULC using simulation modeling for the Malacca River watershed.

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Materials and Methods

Study Area

Malacca state is located in southwest Peninsular Malaysia at 2°23'16.08"N to 2°24'52.27"N latitude and 102°10'36.45"E to 102°29'17.68"E for longitude. Malacca state has a catchment area of approximately 670 km^2 and contains an 80 km length of Malacca River flowing through Alor Gajah and Malacca Central (*Figure 1*). Malacca state also has a reservoir located between Alor Gajah and Malacca Central called the Durian Tunggal Reservoir; with a catchment of 20 km^2. This reservoir supplies water to Malacca residents. Increasing local population has led to increasing public facilities such as transport, healthcare, accommodation, sewage and water supply services (Hua, 2017; Rosli et al., 2015). Due to drastic population growth, rapid urban development in Malacca state also increased; especially demanding from the land use perspective. A majority of residential activities are centralized in the city, which extends about 10 km to the west, the east, as well as the north for 20 km. Land use changes is continuously develop until today. Indirectly, these actions have contributed to economic growth and social relationships, including impacting the environmental quality of the water in the Malacca River.

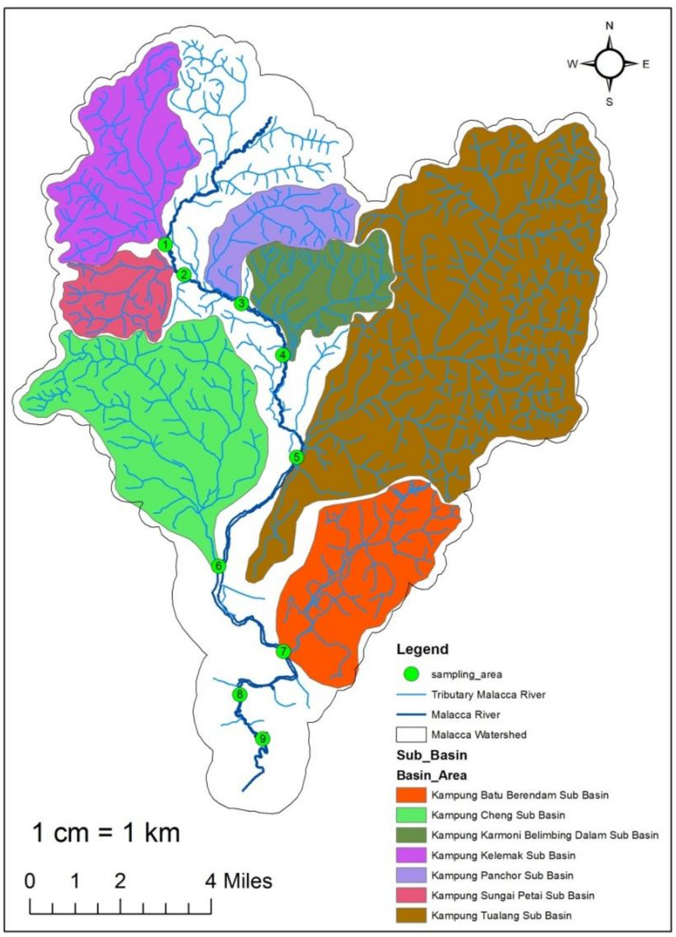

Figure 1. *The catchment area with the river flow in Malacca state*

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Data Collection

Remotely sensed data have been widely used as a very cost effective mean to obtain geo-referred data and maps for evaluation and monitoring LULC. Three LULC data sets of the Malacca River watershed were obtain from USGS Earth Explorer dated 2001, 2008, and 2015 and used in this study. These three data sets are considered suitable with cloud-free spatial coverage and relatively high spatial and spectral resolution of satellite images. Remote sensing images of Landsat satellites were selected for the investigation of long-term variations in LULC types in the study area (*Table 1*). After the limitations and constraints regarding of the data were taken into account, a 14-years period or time-span was found appropriate for monitoring and evaluation of LULC dynamic. The steps of the research study are shown in *Figure 2*.

Table 1. *Landsat data sources*

Satellite	Sensor	Acquisition	Band Combination
Landsat 4-5	TM	2001	1,2,3
Landsat 7	ETM+	2008	1,2,3
Landsat 8	OLI	2015	2,3,4

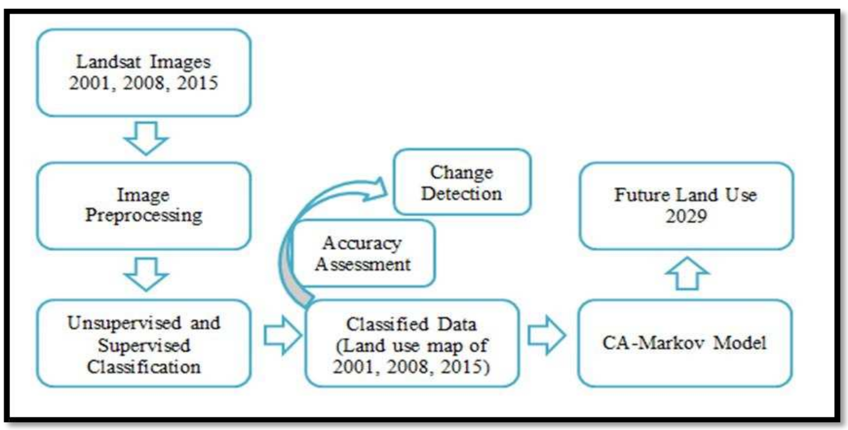

Figure 2. *Demonstration of research methodology as a flowchart. The 2001 and 2008 maps used to simulated 2015 maps and the simulated 2015 map was used to validate the model. Then 2008 and 2015 maps were use as the input to produce simulated 2029 map*

Data Pre-Processing and Image Classification

Preprocessing of satellite images is essential and aims at the unique goal of establishing a more direct linkage between data and the biophysical phenomena it represents (Parsa et al., 2016). Pre-processing is accomplished using ArcGIS version 10.0 for geo-referencing, mosaicking and sub setting of the image for the Area of Interest (AOI). Landsat 8 image underwent spatial sharpening using the panchromatic bands, which resulted in a 15m resolution. Meanwhile, Landsat 5 TM and Landsat 7 ETM+ images for 2001 and 2008 were in original 30m resolution. Further image processing analysis was carried out using

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ENVI 4.0. The image was displayed in natural color composite using a band combination of 3, 2, 1 for Landsat 5 TM and 4, 3, 2 for Landsat 8. Maximum Likelihood supervised classification was performed using several selected regions, and Regions of Interest (ROI) were based on delineated classes of vegetation area, industrial and non-industrial area, water, open space area and farming area (*Table 2*).

Table 2. *Classes delineated on the basis of supervised classification*

Class Name	Description
Vegetation Area	Includes all agricultural lands and forest fields.
Non-Industrial Area	Includes all residential, commercial, administration, cemetery and transportation, as well as sewage treatment plant (include individual septic tank).
Industrial Area	Includes all industrial area.
Water Bodies	Includes all water bodies (river, lakes, gravels, stream, canals, and reservoirs).
Open Space Area	Includes all land areas that exposed soil and barren area influenced by human.
Farming Area	Includes all animal husbandry (or farming) area.

Accuracy Assessment

Accuracy assessments for the 2001, 2008, and 2015 images were carried out to determine the quality of information provided from the data. If the data are to be used for change detection analysis, it is important to conduct accuracy assessment for individual classification (Behera et al., 2012). Kappa tests are used to measuring the accuracy of classification as the test is able to account all elements in confusion matrix including diagonal elements (Halmy et al., 2015). The Kappa test is a measure between predefined producer rating and user assigned rating, which can be expressed in the formula as:

$$K = \frac{P(A) - P(E)}{1 - P(E)} \quad \text{(Eq. 1)}$$

where *P(A)* is the number of time the *k* raters agree, and P(E) is the number of time the *k* raters are expected to agree only by chance (El-Kawy et al., 2011; Pontius and Millones, 2011). Meanwhile, user accuracy can be defined as the probability of a pixel on the image actually representing a class on the ground. Producer’s accuracy indicates the probability a pixel being correctly classified and is mainly used to determine how well an area can be classified (Pontius and Millones, 2011). As described earlier, the six categories of classes which include vegetation area, non-industrial area, industrial area, water bodies, open space

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area, and farming area, that have been delineated should have a minimum of 50 points for each considered category to increase the percentage of accuracy assessment (El-Kawy et al., 2011). Therefore, the accuracies of classification for 2001, 2008 and 2015 are 89.51%, 88.49%, and 92.21%, which have kappa statistics of 0.87, 0.85 and 0.90 respectively. According to Weng (2010), the minimum level for accuracy assessment in identification of LULC categories in remote sensing data should be at least 85%. Afterwards, the data is exported into an ASCII text file to enable for further analysis in ArcGIS version 10.

LULC Change Detection Analysis

In performing LULC change detection, the post-classification detection method is applied in the IDRISI Selva environment v.17, which involves two classified images to make a comparison to produce change information on a pixel basis. In other words, the interpretation between two image provide will provide changes “-from, -to” information. Classified images from two different data sets are compared using cross-tabulation in determining qualitative and quantitative aspects of changes for periods from 2001 to 2015. The magnitude of change and percentage of changes can be expressed in a simple formula as follows:

$$K = F - I \quad \text{(Eq. 2)}$$

$$A = \frac{(F - I)}{I} x100 \quad \text{(Eq. 3)}$$

where K is magnitude of changes, A is percentage of changes, F is first data, and I is reference data (Mahmud and Achide, 2012). Additional, prediction or estimation of LULC changes for 2029 will also use IDRISI Selva environment v.17. This research study uses LULC techniques in remote sensing to determine differences and define the percentage of land use changes within that time, as well as estimation for the next 14 years.

Markov Chain Model Analysis

The Markov chain model was presented by a Russian mathematician named Andrei A. Markov in 1970. This model was first used by Burnham for land use modeling (Mishra and Rai, 2016; Parsa et al., 2016). Markov chains are stochastic processes (Halmy et al., 2015; Subedi et al., 2013) and the matrices to show changes between land use categories (based on the basic core principle of continuation of historical development) (Koomen and Borsboom-van Beurden, 2011) and are often used in modeling and simulation changes and trends of LULC (Halmy et al., 2015; Mishra and Rai, 2016; Parsa et al., 2016). The homogeneous Markov model for prediction of land use changes can be mathematically presented as follows (Subedi et al., 2013):

$$L_{(t+1)} = P_{ij} \times L_{(t)} \quad \text{(Eq. 4)}$$

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$$P_{ij} = \begin{bmatrix} P_{11} \; P_{12} & P_{1m} \\ P_{21} \; P_{22} & P_{2m} \\ \\ P_{m1} \; P_{m2} & P_{mm} \end{bmatrix} \quad \text{(Eq. 5)}$$

where $L_{(t)}$ and $L_{(t+1)}$ represent land use status at time t and $t + 1$ respectively. Including $\sum_{j=1}^{m} P_{ij} = 1(i, j = 1,1,2, \quad .m)$ is the transition probability matric in a given state. Practically in IDRISI Selva v.17, this study uses 2001 and 2015 map into Markov chain model to produce the transition matrix changes between the current 14 years, and the process is repeated onto map 2015 and 2029 for future land use to derive the transition matrix changes.

Cellular Automata (CA)

Cellular Automata (CA) was developed by Ulam in 1940 (Mishra and Rai, 2016; Parsa et al., 2016) for application in land use changes conceptually. Afterwards, CA was used by Tobler in geographical modeling (Arsanjani et al., 2013), and widely used in spatial model (Halmy et al., 2015) for forecasting future land use. CA consists of a grid or a raster space, a set of states characterizing the grid cells and a definition for the neighborhood arrangement of cells, a set of transition rules determine the state transitions for each of the cells as a function of the position of neighboring cells and a sequence of discrete time steps then updates composition and configuration of all the cell simultaneously (Arsanjani et al., 2013; Mishra and Rai, 2016; Parsa et al., 2016). The basic principle of CA is that the land use changes for any location (cells) can be explained by the current state and changes in neighboring cells (Koomen and Borsboom-van Beurden, 2011).

CA-Markov Chain Model

The ‘Cellular Automata’ and ‘Markov Chain’ models are considered to be advantageous for modeling land use changes (Mishra and Rai, 2016; Parsa et al., 2016). The issues involved when a Markov chain model lacks spatially referred output and transition probabilities may be accurate on a categorical basis, there are no specifications on spatial distribution of each land use category occurrence (Arsanjani et al., 2013). Cellular automata added into a Markov model lead to probable spatial transitions occurring in particular area over a period time (Subedi et al., 2013). In other words, the quantity of changes from the Markov Chain model then are made geo-referred and spatial through cellular automata (Mishra and Rai, 2016). The CA-Markov model uses Markov Chain analysis outputs, particularly the Transition Area file, to apply a contiguity filter to enable the development of other land use characteristics from time two into a later time period (Parsa et al., 2016). The CA is able to develop a weighting spatial on the particular areas which have approximately the same to the existing land use based on classes, and it is not random (Subedi et al., 2013). Hence, the CA-Markov model is considered a robust approach because of the quantitative estimation and the spatial and temporal dynamic it has for modeling the LULC dynamic (Arsanjani et al., 2013; Mishra and Rai, 2016; Parsa et al.,

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2016; Subedi et al., 2013). GIS and remote sensing is easy to fir with CA-Markov model to facilitate tasks and reduce the cost and time needed (Mishra and Rai, 2016; Parsa et al., 2016). In this study, IDRISI Selva v.17 is used to predict the future LULC of study area on CA-Markov model. In other words, CA-Markov will use the 2001 and 2008 maps to produce a simulated 2015 map, which is important to validate with actual LULC of 2015 map through KIA (Kappa Agreement of Index) approach (Mishra and Rai, 2016; Parsa et al., 2016). Afterwards, the techniques are repeated using the 2008 and 2015 map to produce a simulated 2029 map.

Validating LULC Prediction Model

In order to avoid miscalculation, investigation between actual image and simulated image will be carried out, where the model's output was compared to a present or actual land use map. Comparing the predicted LULC map representing the 2015 LULC with actual LULC (map of 2015) was based on Kappa Index of Agreement (KIA) approach, which is widely used in validate LULC change predictions (Mishra and Rai, 2016; Parsa et al., 2016; Subedi et al., 2013). An additional map of Landsat TM 2008 will be used to help in validating process, before CA-Markov model can be applied for estimation of the next 14 years. The validation module is available in IDRISI Selva environment v.17 for this purpose.

Results

LULC maps assessment

The images show that the LULC maps significantly change from one class to another class (*Figure 3*). Based on general observation, drastic changes can be detected on water bodies between 2001 to 2015 [*Figure 3 (a)* and *(c)*], as most of these classes change from blue into green color and the area becomes smaller-scale. In other words, the water bodies coverage is suspected to transform into vegetation area, which refers as agriculture activities that carried out within the reservoir. Meanwhile, the non-industrial area is suspected to increase by a big margin, especially in the urban area. Majority classes that converted into non-industrial area are vegetation area and open space area. Only the small scale is subjected to the changes that occur in sub-urban and rural areas. At the same time, small scale shows an increase in industrial area, which is likely to be converted from open space area.

Continuously, the LULC map changes from 2015 to 2029 indicate that majority activities are converted from vegetation area into non-industrial and industrial area. At the same time, several areas on open space classes are also having converted into non-industrial area. The non-industrial area having big margin in conversion of development, followed by industrial area that have partially big scale in increasing the area for development purposes. Most of the development is occur in sub-urban and rural area. This rapid development shows positive impact towards the urbanization and modernization in Malacca state.

Validating LULC prediction model

In order to validate the LULC prediction given by the CA-Markov model, the simulated land use areas were used to compare the actual present land use areas. The LULC for 2015

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was predicted through the model to produce simulated map and make comparison with the actual LULC map of 2015. Comparison of simulated and classified map for the year 2015 can be shown in *Table 3*. Visual analysis shows that simulated LULC map and actual map have relatively close resemblances.

Table 3. *Comparison of actual and projected LULC types in 2015*

LULC type	Area (ha)	
	Actual map	Simulated map
Vegetation Area	39650	39873
Non-Industrial Area	21370	20852
Industrial Area	2660	2628
Open Space Area	1348	1689
Water Bodies	1103	1104
Farming Area	869	854

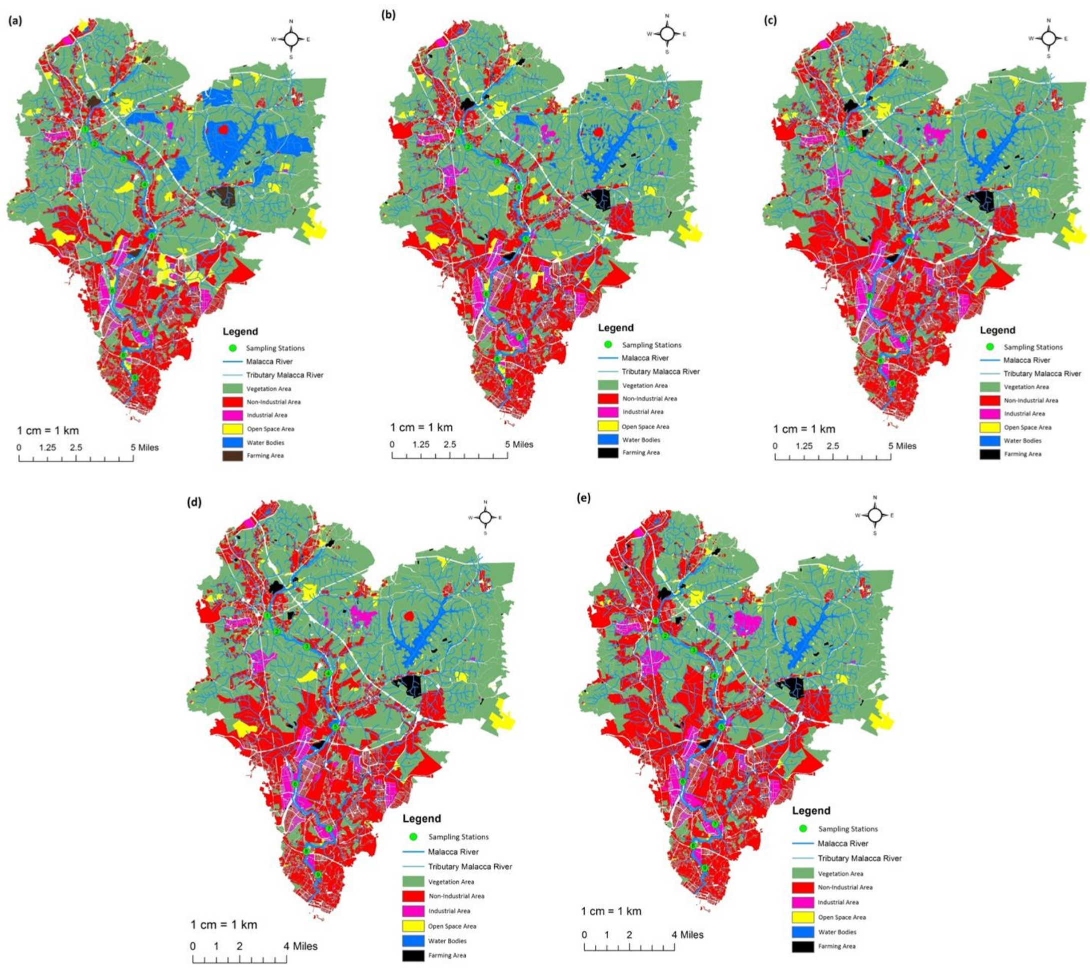

Figure 3. *LULC actual map of (a) 2001, (b) 2008 and (c) 2015. LULC simulated map of (d) 2015 and (e) 2029*

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Table 3 indicates that industrial area, water bodies, and farming area have the best agreement. The simulated areas are 2628 ha, 1104 ha and 854 ha, while the actual area is 2660 ha, 1103 ha and 869 ha respectively. The simulated LULC map shows that non-industrial area, industrial area, and farming area are underestimated; while vegetation area, open space area, and water bodies are overestimated. Hence, the detailed statistical analysis based on the Kappa coefficient is used to measure the overall agreement of matrix, the ratio diagonal values summation versus total number of pixel counts within matrix, and the non-diagonal elements will be the best approach to assess the model accuracy (Arsanjani et al., 2013).

A kappa value of 0 illustrates the agreement between actual and reference map (equals chance agreement), the upper and lower limit of kappa is +1.00 (its occur when the is total agreement) and -1.00 (its happen when agreement is less chance (Arsanjani et al., 2013). The accuracy assessment process was done using VALIDATE module in IDRISI Selva environment v.17. The results indicate K values (Kno = 0.878; Klocation = 0.863; KlocationStrata = 0.863; Kstandard = 0.843) above 0.8 showing satisfactory level of accuracy. According to Viera and Garrett (2005), if the results are greater than 0.8 for each kappa index agreement, then the K statistics are considered accurate. Therefore, CA-Markov modeling is suitable for accurate prediction of future LULCs. This may be useful in this study for environmental management decision making and planning which involve the water quality of Malacca River.

LULC change detection

LULC change can be described in *Table 4*, where the activities carry out in Malacca River basin can be classes into vegetation area, non-industrial area, industrial area, open space area, water bodies, and farming area. According to LULC changes between 2001 to 2008 (*Table 5*), vegetation area and open space area are likely to be transform into non-industrial area (with 22.36% and 24.31%) and industrial area (with 3.13% and 2.04%) respectively. Water bodies is suspected to be decline about 66.57% and change into vegetation area, where this situation happen due to the sufficient water supply and fertile soil leads to the agriculture activities could be carried out. Based on the *Figure 3 (a)* and *(b)*, the agriculture activities are likely to occur in north-east area especially surrounding the reservoir. Meanwhile, open space area is also subjected to transform into vegetation area with 7.07%. Only a small percentage is detected to convert from vegetation area into farming area with 0.05%, indicating that a minority of animal husbandry is carried out on a small scale and mostly being conducted by local residents.

Table 4. *Area (ha) of LULC type in Malacca River basin for 2001, 2008, 2015, and 2029*

LULC type	**Year**			
	2001	2008	2015	2029
Vegetation Area	41841	41391	39650	35755
Non-Industrial Area	15829	18915	21370	24759
Industrial Area	1763	2173	2660	3313
Open Space Area	2695	2115	1348	1237
Water Bodies	4075	1603	1103	1025
Farming Area	797	803	869	911

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Table 5. *Transition probability of area and matrix calculated using land-use maps of 2001-2008*

Category of LULC		**2001-2008**					
		VA	**NIA**	**IA**	**OSA**	**WB**	**FA**
Vegetation Area	F P	30987274 74.46	93055338 22.36	1302581 3.13	0 0.00	0 0.00	20808 0.05
Non-Industrial Area	F P	521160 3.00	14766200 85.00	521160 3.00	521160 3.00	521160 3.00	521160 3.00
Industrial Area	F P	0 0.00	0 0.00	1672800 85.00	295200 15.00	0 0.00	0 0.00
Open Space Area	F P	170033.5 7.07	584655.5 24.31	49062 2.04	1601249 66.58	0 0.00	0 0.00
Water Bodies	F P	1889922 66.57	0 0.00	0 0.00	0 0.00	949077.7 33.43	0 0.00
Farming Area	F P	24000 3.00	24000 3.00	24000 3.00	24000 3.00	24000 3.00	680000 85.00

VA means Vegetation Area; NIA means Non-Industrial Area; IA means Industrial Area;
OSA means Open Space Area; WB means Water Bodies; FA means Farming Area;
F means Frequency (ha); P means Percentage

The demand for development is detected to increase for the next 7 years, from 2008 to 2015. This situation spurred by the recognition of Malacca state as a historical tourism by UNESCO in 2007, where the condition increasing the development activities especially involve with non-industrial area and industrial area [*Table 6* and *Figure 3(c)*]. For example, commercial activities such as restaurants, shopping center, and hotels will be built up in a big margin to provide adequate services for tourists. Directly, vegetation area will continuously decline into non-industrial area (with 7613802 ha), industrial area (with 1430374 ha), and open space area (with 4052.05 ha). At the same time, the open space area will also become a ‘victim’ of development by transforming into non-industrial area (with 634941 ha) and industrial area (with 158432.3 ha). The development of industrial and commercial activities provides job opportunities, attracting the people to centralize in urban areas of Malacca state. Hence, migration of local residents from rural areas and non-local residents into the Malacca state has increased the development activities that boosted into sub-urban area in providing residential area to the people. Indirectly, water bodies coverage will continue being pressure to be decline in quantity in supplying area to vegetation with 560683.2 ha; while farming area is subjected to be increase equivalent with non-industrial and industrial area, which converted from vegetation area for 283643.5 ha. Most of the farming area is referring to the animal husbandry activities.

Table 6. *Transition probability of area and matrix calculated using land-use maps of 2008-2015*

Category of LULC		**2008-2015**					
		VA	**NIA**	**IA**	**OSA**	**WB**	**FA**
Vegetation Area	F	31188629	7613802	1430374	4052.05	0	283643.5
	P	76.97	18.79	3.53	0.01	0.00	0.70
Non-Industrial Area	F	604275	17121125	604275	604275	604275	604275
	P	3.00	85.00	3.00	3.00	3.00	3.00
Industrial Area	F	0	387848	2028652	0	0	0
	P	0.00	16.05	83.95	0.00	0.00	0.00
Open Space Area	F	0	634941	158432.3	938126.7	0	0
	P	0.00	36.67	9.15	54.18	0.00	0.00
Water Bodies	F	560683.2	0	0	0	792316.8	0
	P	41.44	0.00	0.00	0.00	58.56	0.00
Farming Area	F	127155.6	0	0	0	0	708844.4
	P	15.21	0.00	0.00	0.00	0.00	84.79

VA means Vegetation Area; NIA means Non-Industrial Area; IA means Industrial Area;
OSA means Open Space Area; WB means Water Bodies; FA means Farming Area;
F means Frequency (ha); P means Percentage

Overall, the continuous development that occur for 14 years began from 2001 to 2015 is non-industrial and industrial area which converted from vegetation area into 11009434.1 ha (27.02%) and 1752056.5 ha (4.30%) respectively; while reducing coverage area is detected from the change of water bodies (2589000 ha) into vegetation area for 1992494.4 ha (76.96%), and open space area (2021500 ha) transform into non-industrial area with 894513.75 ha (44.25%), industrial area with 162528.6 ha (8.04%), and vegetation area with 106533.05 ha (5.27%) (*Table 7*). Meanwhile, the simulated future LULC changes for 14 years from 2015 to 2029 indicate that the statistics for non-industrial area, industrial area, and farming area continue to climb (*Table 8*). The vegetation activities keep 'donating' area into non-industrial area with 25.97%, industrial area with 4.13%, and farming area with 0.56%. At the same time, open space activities are transforming into non-industrial area at 17.67% and industrial area at 4.24%. Water body coverage will be pressured by vegetation area for 34.36% or 365590 ha to provide agricultural activities for local residents.

Table 7. *Transition probability of area and matrix calculated using land-use maps of 2001-2015*

Category of LULC		**2001-2015**					
		VA	**NIA**	**IA**	**OSA**	**WB**	**FA**
Vegetation Area	F	27800654.65	11009434.1	1752056.5	0	0	183354.75
	P	68.23	27.02	4.30	0.00	0.00	0.45
Non-Industrial Area	F	557985	15809575	557985	557985	557985	557985
	P	3.00	85.00	3.00	3.00	3.00	3.00
Industrial Area	F	0	359147.6	1852352.4	0	0	0
	P	0.00	16.24	83.76	0.00	0.00	0.00

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Open Space Area	F P	106533.05 5.27	894513.75 44.25	162528.6 8.04	857924.6 42.44	0 0.00	0 0.00
Water Bodies	F P	1992494.4 76.96	0 0.00	0 0.00	0 0.00	596505.6 23.04	0 0.00
Farming Area	F P	126782.6 15.22	0 0.00	0 0.00	0 0.00	0 0.00	706217.4 84.78

VA means Vegetation Area; NIA means Non-Industrial Area; IA means Industrial Area;
OSA means Open Space Area; WB means Water Bodies; FA means Farming Area;
F means Frequency (ha); P means Percentage

Table 8. *Transition probability of area and matrix calculated using land-use maps of 2015*-2029

Category of LULC		2015-2029					
		VA	**NIA**	**IA**	**OSA**	**WB**	**FA**
Vegetation Area	F P	26142914 69.34	9791339 25.97	1557113 4.13	0 0.00	0 0.00	211134 0.56
Non-Industrial Area	F P	0 0.00	19531019 84.68	3533481 15.32	0 0.00	0 0.00	0 0.00
Industrial Area	F P	89595 3.00	89595 3.00	2538525 85.00	89595 3.00	89595 3.00	89595 3.00
Open Space Area	F P	0 0.00	228385 17.67	54802 4.24	1009313 78.09	0 0.00	0 0.00
Water Bodies	F P	365590 34.36	0 0.00	0 0.00	0 0.00	698410 65.64	0 0.00
Farming Area	F P	111517 12.53	0 0.00	0 0.00	0 0.00	0 0.00	778483 87.47

VA means Vegetation Area; NIA means Non-Industrial Area; IA means Industrial Area;
OSA means Open Space Area; WB means Water Bodies; FA means Farming Area;
F means Frequency (ha); P means Percentage

LULC changes trends can be described as the vegetation area transforming into open space area before it converted into non-industrial area, industrial area, and farming area. Meanwhile, water bodies are likely to change into vegetation area. Therefore, the LULC changes trends can be express in the net changes for 2001 to 2015, which indicate that non-industrial area, industrial area, and farming area have high positive values of 9473170.45 ha, 2113422.5 ha, and 614557.15 ha respectively; while vegetation area, open space area, and water bodies have high negative values with -10161050.3 ha, -605590.4 ha, and -1434509.4 ha respectively [(*Table 9* (*i*)]. For the next 14 years between 2015 to 2029 [(*Table 9 (ii*)], only industrial area has high increment with 2583998.5 ha to resulted as 4697421 ha, while non-industrial area and farming area remain positive for 6575838 ha and 189212 ha respectively. Lastly, the net changes for open space area and water bodies remain negative by -193592 ha and -275995 ha, while vegetation area continue to decrease by -10992884 ha.

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Table 9 (i). *Rate of losses, gains, and net changes of LULC areas (ha) for 2001 to 2015*

LULC types	**2001-2015**		
	L	G	NC
VA	-12944845.35	2783795.05	-10161050.3
NIA	-2789925	12263095.45	9473170.45
IA	-359147.6	2472570.1	2113422.5
OSA	-1163575.4	557985	-605590.4
WB	-1992494.4	557985	-1434509.4
FA	-126782.6	741339.75	614557.15
Total	-19376770.35	19376770.35	0

L means Losses; G means Gains; NC means Net Changes; VA means Vegetation Area;
NIA means Non-Industrial Area; IA means Industrial Area; OSA means Open Space Area;
WB means Water Bodies; FA means Farming Area

Table 9 (ii). *Rate of losses, gains and net changes of LULC areas (ha) for 2015 to 2029*

LULC types	**2015-2029**		
	L	G	NC
VA	-11559586	566702	-10992884
NIA	-3533481	10109319	6575838
IA	-447975	5145396	4697421
OSA	-283187	89595	-193592
WB	-365590	89595	-275995
FA	-111517	300729	189212
Total	-16301336	16301336	0

L means Losses; G means Gains; NC means Net Changes; VA means Vegetation Area;
NIA means Non-Industrial Area; IA means Industrial Area; OSA means Open Space Area;
WB means Water Bodies; FA means Farming Area

Discussion

The Markov chain model becomes an important model to describe probability movements of an individual in a system comprised of discrete states. When applying in land used perspective, Markov chains are often used to specify both time and a finite set of states as discrete values (Subedi et al., 2013). Transitions between the states of the system are recorded in the forms of a transition matrix that recorded the probability of moving from one state to another state (Behera et al., 2012). Applications of Markov chains in urban and non-urban land use began in the 1970s as an alternative to the use of a large-scale area in simulation models for land use forecasting (Arsanjani et al., 2013). Advantage in determining the development trend and benefit in predicting the state of future leading the Markov chain model are continuously applied in various research studies. As proved, the model's applicability and feasibility had been testified in many research papers and its results approximately conform to observed results (Arsanjani et al., 2013; Behera et al., 2012; Sinha and Kumar, 2013). Therefore, the Markov model result is a transition matrix which shows the probability of changes from each class of LULC to another class in the

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future, which can be described in *Table 8* as transitional probability matrices and as a prediction of LULC statistic for year 2029.

Built-Up Area

Previous studies on water resources through statistical analysis indicate the source of pollutants for river water contamination can involve with residential activities, septic tank and sewage treatment plant activities, farming activities (or animal husbandry activities), and industrial activities; which can be grouped together to classified as built-up area to bring negative impact to the Malacca River (Hua, 2017; Hua et al., 2016). According to Hua (2017) and Rosli et al (2015) stated that Malacca River water quality is detected pollution by physico-chemical and biological parameters, which occur due to major source of pollutants from residential area, industrial area, and agricultural activities. These activities are carried out in urban and sub-urban area. On the other hands, LULC changes from the year 2001 to 2008 and 2008 to 2015 signify the demands towards built-up area category are continuously increasing. In other words, larger scale usage of the LULC area for human activities could increase the percentage of river pollution through the contribution of pollutant sources. Indirectly, increasing demand for land used will develop various categories of activities, which will increase the contamination in the Malacca River. Simultaneously, future land used statistic indicate 11462471 ha (70.32%) in continue 'add-on' for built-up area will estimate the possibilities of river water pollution to changes from clean to slightly polluted or slightly polluted into polluted condition.

Agriculture Land

According to the statistical analysis, agriculture activities are confirmed to be categorized as one element to contribute as a pollutant source. As additional evidence, although LULC changes in agriculture perspective are decreasing from 2001 to 2008 and increasing in a small percentage from 2008 to 2015 (*Table 5* and *Table 6*); however, larger fertilizer and chemical pesticide usage in agriculture activities will contribute non-point source pollution through surface runoff towards the Malacca River. The main focus on development of agricultural land happened in 2008; majority of water category was converted into open space as a preparation for agricultural activities. When the prediction of land used in 2015 to 2029 is estimated, possibility agricultural lands will conquest the water coverage area due to the nature of fertile soil. Moreover, the demands for built-up activities development in 'north-west' area are higher percentage, which may cause the agriculture land to shift from 'north-west' area into 'north-east' area. Nevertheless, even agriculture activities are estimated to reduce in land used area for about -11559586 ha (70.91%), it is not possible to prevent further contamination to occur in the Malacca River.

Water Coverage and Open Space Area

Continuously decrement of water coverage indicates that the area are being converted into an open space or agricultural land as seen in changes from the year 2001 to 2008 with 1889922 ha (66.57%) and 2008 to 2015 with 560683.2 ha (41.44%). As described previously, the water category is being transformed into other activities due to the higher fertility soil for farming, potentially for building construction, widen facilities and services,

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and so on. Nevertheless, the reduction in percentage of water areas not only bring negative impacts; as the means of fresh water supplies and food source, but also affects the ecosystem, especially aquatic animals and contribute contamination through human activities of the point source and non-point source pollution. Future prediction of LULC classes estimates water coverage area is probably negative about 365590 ha (2.24%), which may possible to increase river water pollution and threatened the ecosystem survivor through endangered the environment.

Compared to open space category in LULC, the increment area for 7 years starting 2001 about 803751 (33.42%), but the total area are not remain static which start to reduce about 793373.3 ha (45.82%) for the next 7 years. The possible reason that caused the LULC change between 2001 to 2015 are the majority open space being transform from the area of water coverage and agriculture land into built up area. The main reason to cause this condition to happen is the human population increase drastically causing demand towards residences, job opportunities, food sources, facilities and services to increase. Indirectly, open space activities would also bring non-point source pollution through rapid surface runoff from soil erosion; and oil, grease and toxic chemicals from driveways and roads. Although there is probability to reduce pollution by reducing open space category between the year 2008 to 2015, but the percentage to increase pollutant sources through various human activities will still continue to happen. This is due to the number of land areas converted into built-up areas. Unfortunately, future prediction for the open space category are continue to be negative about 283187 (1.73%), which contribute to the land used activities or build-up area that serve the interest of the people without awareness of environment that would probably continuous to contaminate the Malacca River.

Conclusion

This study has shown the important role of LULC in Malacca River watershed in providing proper information in time for decision making concerning the degradation of river water quality. The above-mentioned method for extraction of LULC maps (2001, 2008, and 2015), and the simulation used to predict the future LULC (2029) aided by the CA-Markov modeling, have allowed the researcher to obtain information from analysis of LULCs in respect to the type and the extent of each LULC, while illustrating LULC conversions for the whole watershed in the study area. Generally, the results of this study indicate that supervised classification of remote sensing images is a robust mean of extracting appropriate LULC maps. The ability of simulation model in prediction proved to be good, while the CA-Markov model is a useful tool for LULC prediction (especially in quantitative and qualitative). Therefore, the CA-Markov model is important to land use policy design and planning especially involve with LULC development, which requires a framework for archiving the goals and objectives of sustainable land use development.

The Malacca River has experienced contamination due to the increasing of population, industrial development, agricultural expansion and forest degradation, and this has resulted to uncontrolled and unmanageable of LULC development. Therefore, continuous development has led to increasing pollution and aquatic ecosystem degradation, biodiversity resources destruction, as well as environmental damage in the watershed.

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Hence, future land use change maps can be used as an early warning system for proper land use development to protect undisturbed area of natural ecosystem and biodiversity from human activities. Simultaneously, a future map could help for planning and management in reducing river contamination by selecting only the land suitable to be developed for economic purposes without disturbing the importance of the environmental perspective.

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DETERMINATION OF FORAGE PRODUCTIVITY, CARRYING CAPACITY AND PALATABILITY OF BROWSE VEGETATION IN ARID RANGELANDS OF CHOLISTAN DESERT (PAKISTAN)

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Abstract. The browse vegetation in Cholistan rangelands of Pakistan have been on decline due to climatic extremes, overgrazing and anthropogenic pressure. Study area is hot arid desert where vegetation grow after rainfall but over grazing, extreme weather, and local comunity pressure for fire wood, timber, and harvesting of plants for varius purposes causing the decline of browse species. Therefore, a study was carried to investigate forage productivity, carrying capacity and palatability of browses. Multiple surveys were conducted from 2011-2012 at twenty range stands and 25 browse species belonging to 12 families were identified. Results revealed that browse productivity was high (8029.1 kg/ha) in wet season as compared to dry season (5422.9 kg/ha), correspondingly carrying capacity was high during wet season (16 ha/AU/Y) than dry season (24 ha/AU/Y). Moreover, during dry season, mostly stands were observed to be overgrazed while in wet season maximum stands were moderately grazed. High carrying capacity and good grazing status of stands in wet season was due to better forage production. Based on palatability classification, 22 species were found to have palatability to varying degree and 3 species were non-palatable. In palatable species, leaves of 14 species; shoot/stem of 13 species, flower of 4 species, and fruit of 3 species were grazed by livestock, whereas cattle were observed to graze on 7 species; goat and sheep like 10 species each while camel prefer 20 species. The findings of this study indicate that overall the browse productivity of Cholistan rangelands was low and vary according to seasonal rainfall. Therefore, protection, suitable stocking rate and planned grazing is vital to conserve browses ultimately for sustainability of Cholistan rangelands.
Keywords: *browse species, biomass, stocking rate, grazing livestock, sustainability*

Introduction

Rangelands cover about 50% of the world’s land surface and are large tracts of natural vegetation which support livestock. Mostly rangelands are in vegetation biomes such as grasslands, shrublands, savannas, and deserts. These areas are often characterized by arid climate with low rainfall, and seasonal temperature extremities. Studying the relationship among rangelands components (livestock and plants) is most important tools to adopt suitable measures for best practical use of range resources

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(Friedel et al., 2000). Rangelands provide vegetation cover and soil protection which ensures sustainable production of forage for livestock. Especially browses (shrubs and tree foliage) beside grasses compose cheap sources of feed for animals in World. The importance of browse species as animal feed is reported especially from harsh environments in arid and semi-arid rangelands. Mostly these species have advantage of maintaining their nutritive value and greenness during dry season when grasses decline in both quantity and quality. This nutritious profusion and perennial performance of browses afford round the year provision of forage for livestock (Mtengeti and Mhelela, 2006).

Rangelands quality largely dictate animal productivity and thus it becomes vital to maintain sustainable feed resources from rangelands without deterioration (Rubanza et al., 2006). The justifiable use of rangelands is vital for the development of national economy. Pakistan is a sub-tropical country, which consists of vast semi-arid and arid tracks of land, stretches over 68 million hectares (Majeed et al., 2002). Pakistan has a wealth of 135 million heads of livestock, which account for 10.8% of the GDP. Livestock sector play very significant role because it provides numerous services for humankind like milk and meat, which are vital components of our diet. Livestock occupies a key position in the rural economy of Pakistan for improving the living standard of small resource peoples (Khan et al., 2005).

The rangelands are degrading due to overgrazing, unscientific livestock management, excessiveness of unpalatable species, change in climate, and disturbance of soil (Landsberg and Crowley, 2004). Previous policies have always supported the crops production over livestock, leading in misuse of lands having economically ineffectual productive potential. The herbaceous vegetation of these rangelands only flourishes in monsoon season; accordingly, livestock herds show pitiable health and produce very poor yield of meat and milk. These problems are common everywhere in the world where arid or semiarid rangelands exist. Therefore, developing countries like Pakistan face the similar situation in their rangelands health. Rangelands, which constitute about 65 % of the total area of Pakistan, are in declining process with the passage of time (Ahmad and Hasnain, 2001).

Furthermore, Loeser et al. (2006) showed that episodic drought interacts with grazing, leading to infrequent but biologically important shift in plant communities and suggested the importance of climatic variation in determining ecological effects of grazing practices. Few studies have attempted to unravel the effects of grazing and drought on desert rangelands. It appeared that negative effects of grazing on natality, mortality, or population turnover can be accentuated for certain species when subject to below average precipitation (Curtin, 2002). Globally, grazing has been one of the key disturbance factors resulting in rangeland degradation, an increase of spatial homogeneity of the rangelands, an alteration of rangelands function, and loss of species diversity (Gamoun, 2014).

The Cholistan rangelands were formerly a thriving and prosperous area but now largely converting into an abandoned patch. The productivity of its rangelands is degrading ultimately, carrying capacity of this area is decreasing (Akhter and Arshad, 2006). Sustainability of life in this hot desert rotates around the annual rainfall. During summer season, weather is tremendously severe and harsh; certain xeric plant species survive but suffer high grazing pressure and leading to partial eradication (Arshad et al., 2008). Resultantly, the palatable species are diminishing and unpalatable species with less nutritious properties are becoming abundant. Continuous increase in human

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population for livelihood and multiplying number of livestock is adding towards the desertification (Abdullah et al., 2013).

Assessing forage productivity and carrying capacity are key factors of rangeland inventory and monitoring programs which are highly required for sustainability of natural resources. The browse species are one of the most important and nutritionally rich sources of feed for livestock in Cholistan rangelands. Due to year-round stress, the browses of Cholistan rangelands are under severe threat and need detail assessment of their potential. Already no conservational measures have been made in Cholistan rangelands because of unavailability of sufficient data. In order to preserve the optimum production of browse species and their justifiable use in future, information about current range resources is very important. Therefore, this study was being planned to collect base line data about productivity of browses, carrying capacity and palatability to chalk out their management strategy in Cholistan rangelands.

Material and methods

Description of study area

The study was conducted in Cholistan desert of Pakistan. Cholistan desert, an extension of the Great Indian Desert, is located in southern Punjab of Pakistan, between 27^o 42' and 29^o 45' north and 69^o 52' and 73^o 05' east (*Fig. 1*). The climate of Cholistan desert is characterized by low and sporadic rainfall. The mean annual rainfall varies from less than 100 mm in the west to 200 mm in the east. Rain usually falls during monsoon (July through September), winter and spring (January through March). Aridity is the most striking feature of the Cholistan desert with wet and dry years occurring in clusters. Cholistan is one of the hottest regions of Pakistan. Temperatures are high in summer and mild in winter. The mean summer temperature (May, June) is 34^{o}C with the highest reaching above 51^{o}C. The vegetation of Cholistan desert is xeric, adapted to extremely high temperature, low moisture contents and increased salinity coupled with wide variation of edaphic factors. Main soil types of Cholistan desert are sand dunes (44%), sandy soils (37%), loamy soils (2%) and saline-sodic clayey soils (17%). Fortunately, several nutritious and drought tolerant species of grasses, shrubs and trees are found naturally in this desert especially after rainfall (Akbar et al., 1996).

Reconnaissance survey and study sites

A reconnaissance survey was conducted in January 2011, in order to have an impression of site conditions, to collect information about accessibility, to do an overview of plant assemblages and to determine the sampling and data collection methods. According to schedule, whole research project was carried for two consecutive years i.e. 2011 and 2012. After going through the topographic map of area followed by frequent visits during initial stages of study, research area was divided into 20 stands to cover the variations of physiognomy and physiography almost over the whole Cholistan desert. The specific stand position was determined by a GPS (Global Positioning System) named Garmin eTrex. The geographic coordinate's latitude, longitude, and altitude were taken from each stand (*Table 1*).

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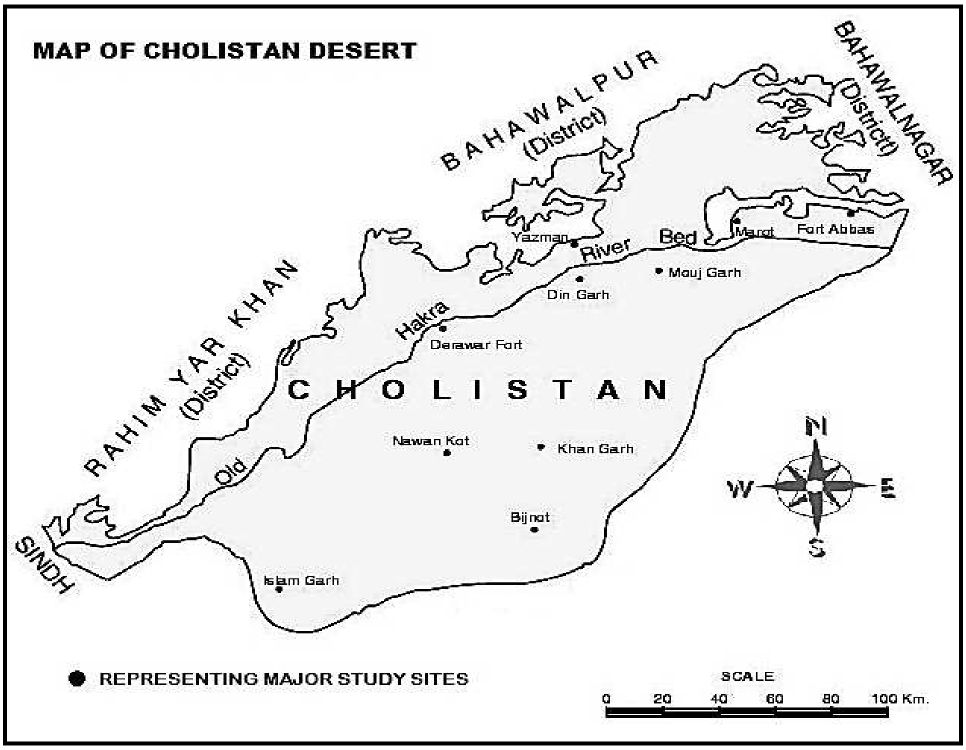

Figure 1. *Map of study area; the Cholistan desert of Pakistan*

Table 1. *Name, location, and topography of each stand in study area*

Sr. No.	Stand Name	GPS Location		Elevation	Topography
1	Marot	N: 29°12.161′	E: 072°15.427′	398 ft	Sandunal
2	Kalapahar	N: 29°10.430′	E: 072°05.569′	384 ft	Clayey saline
3	Sulleh Wala	N: 28°40.315′	E: 071°35.648′	389 ft	Interdunal sandy
4	Januwali	N: 29°05.056′	E: 072°09.933′	406 ft	Interdunal sandy
5	Khirsir	N: 29°10.339′	E: 072°08.749′	391 ft	Sandunal
6	Haider wali	N: 29°02.672′	E: 072°10.200′	382 ft	Clayey saline
7	Mojgarh Fort	N: 29°01.059′	E: 072°08.106′	392 ft	Sandunal
8	Khangarh	N: 28°57.261′	E: 072°03.089′	369 ft	Interdunal sandy
9	Khanser	N: 28°59.227′	E: 071°55.299′	352 ft	Sandunal
10	Bijnot	N: 28°47.988′	E: 071°45.770′	340 ft	Interdunal sandy
11	Dingarh Fort	N: 28°57.454′	E: 071°51.910′	365 ft	Clayey saline
12	Rukanpur	N: 28°53.182′	E: 071°46.362′	371 ft	Sandunal
13	Nidamwala Toba	N: 28°52.963′	E: 071°44.270′	355 ft	Clayey saline
14	Nawankot	N: 28°47.939′	E: 071°45.770′	334 ft	Interdunal sandy
15	Lakhan	N: 28°52.232′	E: 071°42.731′	351 ft	Clayey saline
16	Chananpir	N: 28°56.832′	E: 071°40.057′	353 ft	Interdunal sandy
17	Baylawala	N: 29°23.466′	E: 071°39.563′	410 ft	Interdunal sandy
18	Derawar fort	N: 29°23.465′	E: 071°39.560′	345 ft	Interdunal sandy
19	Chasma Dhar	N: 28°39.864′	E: 071°15.632′	323 ft	Clayey saline
20	Islamgarh Fort	N: 27°50.208′	E: 071°48.129′	334 ft	Sandunal

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Browse forage production

Biomass is a commonly measured vegetation attribute that refers to weight of plant material within an area. Only plants that are available and palatable to grazing animals are classified as forage. Data was collected in wet (August) and dry season (April) both. Biomass was calculated by Direct Harvest method using 100-meter line transect with 1x1 meter square quadrate. At each stand five transect were laid out and quadrate were placed systematically at 10-meter interval on each transect. Clipping was done at grazed-height, because it gives more pertinent measure of forage biomass. The harvested material was packed and labeled in paper bags immediately and weighed in the field to get fresh weight and then oven dried at 65°C for 72 hours in laboratory and re weighed. The dry weight of all quadrates was then combined and averaged to get total dry matter production (kg/ha), at each stand. Grazing status was estimated by direct observation at each stand and categorizes them as overgrazed, moderately grazed, slightly grazed and no grazing (Bonham, 1989; Holechek et al., 1995).

Range carrying capacity

Carrying capacity is an important management tool that connects forage supply with forage consumption. Carrying capacity was estimated based on 40% allowable grazing material. The one animal unit (AU) was taken as, a cow having 350 kg weight, demanding 7 kg dry matter forage per day, 2555 kg/year (Bonham, 1989).

$$\text{Carrying capacity (ha/AU/Year)} = \frac{\text{Animal forage requirement kg /year}}{\text{Forage production kg /ha}} \qquad \text{(Eq.1)}$$

Degree of palatability

Classification of browse species based on palatability, parts used and animal's preferences was recorded by direct observing the grazing livestock (cattle, sheep, goats and camel) in field for two consecutive years. These field observations were further confirmed from knowledge gathered from graziers and nomadic peoples at different range sites of Cholistan desert. In order to calculate the degree of palatability, following palatability classes were used:

i) Highly palatable ii) Moderately palatable iii) Less palatable iv) Non-palatable

The palatable species were classified into four categories based on parts used by livestock:

i) Leave grazed ii) Shoot grazed iii) Flower grazed and iv) Fruit grazed

The livestock mostly differ in their selection of browsing species at different range sites. In present case, browsing species were classified whether grazed by cattle, sheep, goat, or camel (Hussain and Durrani, 2009).

Statistical analysis

Microsoft Excel spreadsheet analysis (MS OFICE, 2010) was conducted to determine simple averages, percentiles and mean values and to make needful tables and graphs (McCullough and Heiser, 2008).

Results

Forage production of browses

Browse forage production was estimated during wet (August) and dry season (April) in the arid rangelands of Cholistan desert. During wet season, total fresh browse productivity from all stands was 14034.6 kg/ha and dry forage productivity was 8029.1 kg/ha. The highest quantity of dry phytomass was attained at stand 8 (554.4 kg/ha) and minimum at stand 20 (321.5 kg/ha). The average fresh forage yield recorded from all stand was 701.73 kg/ha and dry forage yield was 401.46 kg/ha. On the basis of habitats, maximum dry matter production (3681.3 kg/ha) was observed at interdunal habitat, followed by sandunal habitat (2298.5 kg/ha) and then clayey saline habitat (2049.3 kg/ha). However, average dry forage production of browses at interdunal habitat was 460.16 kg/ha followed by sandunal habitat with 383.08 kg/ha then clayey saline habitat with 341.55 kg/ha (*Fig. 2*). Based on grazing intensity in wet season, maximum stands (45%) were observed to be moderately grazed followed by overgrazed (40%) and then slightly grazed (15%) (*Table 2*).

In Cholistan rangelands during dry season the total fresh biomass productivity was 8865.8 kg/ha while dry matter production was 5422.9 kg/ha. The average fresh productivity of all stands was 443.29 kg/ha and dry matter was 271.145 kg/ha. The maximum dry matter of browses was attained at stand 4 (376.5 kg/ ha) while minimum was at stand 20 (161 kg/ha). Along habitats, maximum dry biomass was recorded at interdunal sandy habitat (2633 kg/ha) followed by sandunal habitat (1496.5 kg/ha) and then at clayey saline habitat (1293.4 kg/ha). The average dry forage productivity of interdunal habitat was 329.13 kg/ha followed by sandunal habitat with 249.42 kg/ha then clayey saline habitat with 215.57 kg/ha (*Fig. 2*). In this season, maximum stands were observed to be over grazed (75%), followed by moderately grazed (25%) and there was no stand without grazing effects (*Table 2*).

The comparison of vegetation condition during wet and dry seasons has been shown in *Figure 3*.

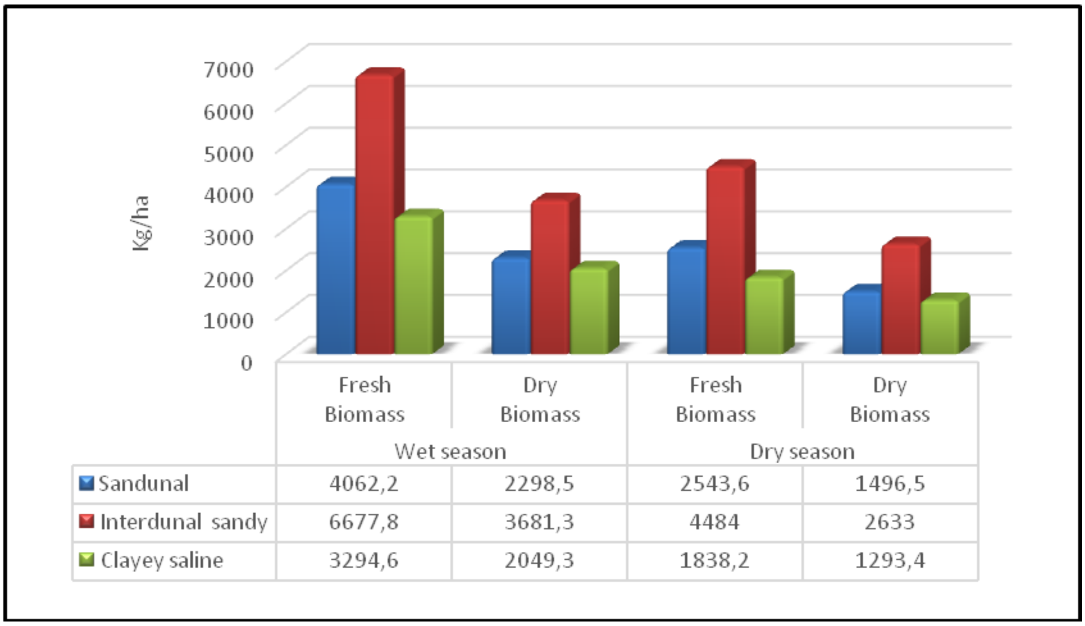

***Figure 2.** Seasonal browse forage yield (kg/ha) at three range habitats in Cholistan*

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Table 2. *Seasonal browse production (kg/ha) and grazing status of each stands*

Stand No.	Topography/Habitat	Season	Fresh biomass	Dry biomass	Grazing status
1	Sandunal area	Wet	669.1	388.2	Overgrazed
		Dry	466	284.8	Overgrazed
2	Clayey saline area	Wet	517.4	328.4	Overgrazed
		Dry	344	221.8	Overgrazed
3	Interdunal sandy area	Wet	793.2	451.2	Moderately grazed
		Dry	577.8	322.5	Overgrazed
4	Interdunal sandy area	Wet	825.6	452.2	Moderately grazed
		Dry	582	376.5	Moderately grazed
5	Sandunal area	Wet	731.7	420.3	Slightly grazed
		Dry	436	244.8	Moderately grazed
6	Clayey saline area	Wet	623	347.7	Moderately grazed
		Dry	335.5	234.5	Moderately grazed
7	Sandunal area	Wet	690.3	365.2	Moderately grazed
		Dry	427.5	240	Moderately grazed
8	Interdunal sandy area	Wet	1017	554.4	Slightly grazed
		Dry	623	354.3	Overgrazed
9	Sandunal area	Wet	650.4	386.8	Overgrazed
		Dry	420.5	232.8	Overgrazed
10	Interdunal sandy area	Wet	920.3	523.7	Slightly grazed
		Dry	625.3	372.8	Moderately grazed
11	Clayey saline area	Wet	593.3	344.8	Moderately grazed
		Dry	327.5	255.5	Overgrazed
12	Sandunal area	Wet	628.6	376.9	Overgrazed
		Dry	402.3	240.8	Overgrazed
13	Clayey saline area	Wet	523.3	367.3	Overgrazed
		Dry	313.9	245.3	Overgrazed
14	Interdunal sandy area	Wet	810	495.6	Moderately grazed
		Dry	607.3	363.5	Overgrazed
15	Clayey saline area	Wet	570	339.6	Moderately grazed
		Dry	266.3	175.3	Overgrazed
16	Interdunal sandy area	Wet	737.4	391.4	Overgrazed
		Dry	493.8	285.3	Overgrazed
17	Interdunal sandy area	Wet	790.3	396.8	Moderately grazed
		Dry	489.3	257.8	Overgrazed
18	Sandunal area	Wet	692.1	361.1	Moderately grazed
		Dry	391.3	253.3	Overgrazed
19	Interdunal sandy area	Wet	784	416	Overgrazed
		Dry	485.5	300.3	Overgrazed
20	Clayey saline area	Wet	467.6	321.5	Overgrazed
		Dry	251	161	Overgrazed

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a b

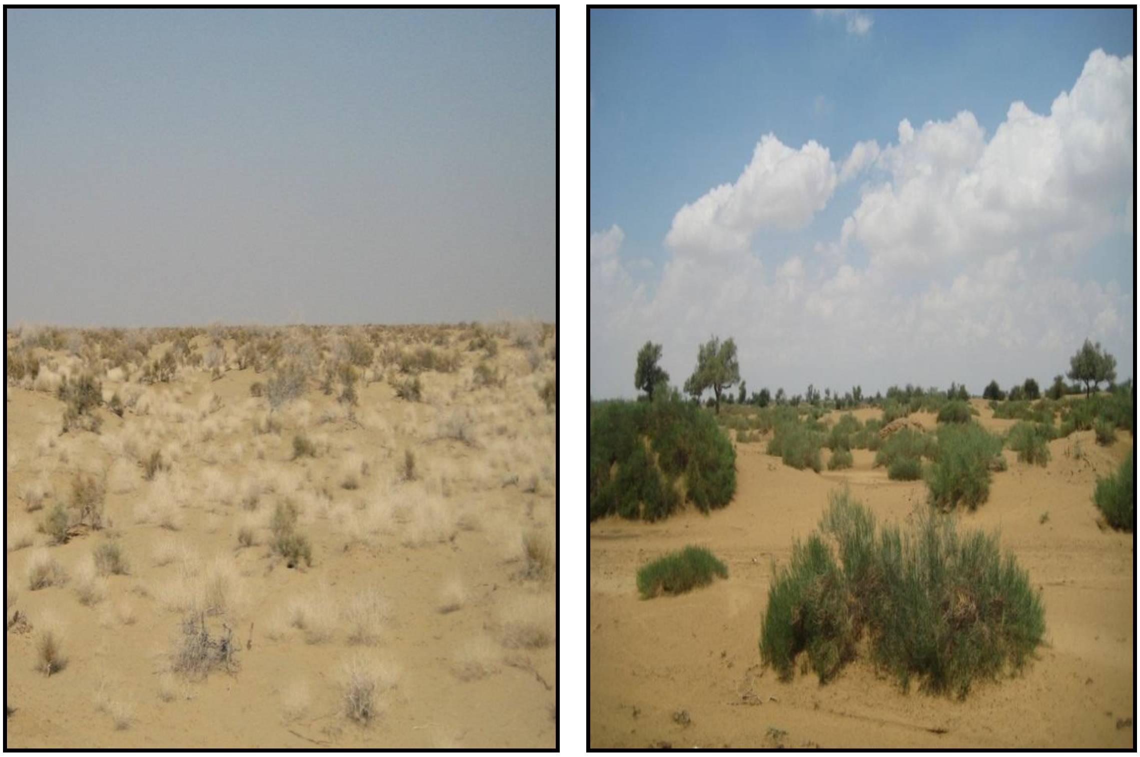

Figure 3. *Vegetation condition in Cholistan desert during a-dry and b-wet seasons*

Range carrying capacity of browses

Carrying capacity or grazing capacity is a common term, used when we are defining the stocking rates. In this study, carrying capacity was calculated in the rangelands of Cholistan desert with respect to browse production. As given in *Table 3* overall average dry browse production during wet season was 394.93 kg/ha while available forage production was 157.97 kg/ha. The proper use factor (PUF) has been taken as 40% to estimate available forage. Over three range habitats in Cholistan desert, maximum available forage was observed at interdunal sandy (184.06 kg/ha) followed by sandunal (153.23 kg/ha) and least at clayey saline (136.62 kg/ha). However, carrying capacity (CC) during wet season was calculated as 16 ha/AU/Y while at interdunal was 14/ha/AU/Y; at sandunal was 17 ha/AU/Y and at clayey saline habitat was 19 ha/AU/Y. It was estimated that Cholistan desert cover an area of 2.6 million hectare out of which 1300000 ha has been considered as rangelands. Based upon this factor, the stocking rate during wet season was calculated as 80376 AU/Y in Cholistan rangelands.

In dry season, overall average dry matter production was 264.71 kg/ha while the available forage was 105.88 kg/ha (*Table 3*). Across the three range habitats in Cholistan desert, maximum available forage was recorded at interdunal habitat (131.65 kg/ha) followed by sandunal habitat (99.77 kg/ha) and then at clayey saline habitat (86.23 kg/ha). Based on available forage overall carrying capacity in dry season was calculated as 24 ha/AU/Y whereas carrying capacity of interdunal was 19 ha/AU/Y, sandunal was 26 ha/AU/Y and at clayey saline habitat was 30 ha/AU/Y. In this season, the stocking density of Cholistan rangelands was calculated as 53872 AU/Y.

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Table 3. *Seasonal carrying capacity (cc) at three range habitats in Cholistan*

Season	**Range habitat**	**Average browse production** (kg/ha)	**Available forage** (kg/ha)	**Carrying capacity** (ha/AU/Y)
Wet season	Sandunal	383.08	153.23	17
	Interdunal sandy	460.16	184.06	14
	Clayey saline	341.55	136.62	19
	Overall average CC	394.93	157.97	16
Dry season	Sandunal	249.42	99.77	26
	Interdunal sandy	329.13	131.65	19
	Clayey saline	215.57	86.23	30
	Overall average. CC	264.71	105.88	24

Palatability classification of browses

Results regarding palatability of browses in Cholistan rangelands revealed that out of total species, 22 (88%) species were found to have palatability to varying degree and 03 (12%) species were found non-palatable. Among palatable species, 7 species (31.82%) were highly palatable, 9 species (40.91%) were moderately palatable, and 6 species (27.27%) were less palatable. In highly palatable class, there were 5 species of trees and 2 species of shrubs. Moderately palatable species were consisting of 2 species of trees and 7 species of shrubs. Less palatable class was composed of 6 species of shrubs while non-palatable class was consisting of only 3 species of shrubs. There were total 7 species of trees in which 71.43% were highly palatable and 28.57% moderately palatable. Out of 18 species of shrubs, 11.11% species were highly palatable, 38.89% were moderately palatable, 33.33% were less palatable, and 16.67% species were unpalatable (*Table 4*).

Palatability by parts used was based on the type of plant parts, used by livestock in Cholistan rangelands. It was observed that among palatable species, leaves of 14 species (63.64%), shoot/stem of 13 species (59.09%), flower of 4 species (18.18%), and fruit of 3 species (13.64%) were grazed by livestock. The first class in which leaves were used, was consisting of 6 species of trees and 8 species of shrubs. In second class in which shoot/stem was used, there were 4 species of trees and 9 species of shrubs. Flower use class, was consisting of 2 species of trees and shrubs each. In fruit class, there were only 3 species of trees (*Table 4*).

According to results out of total palatable browse species, 7 species (31.82%) were grazed by cattle, which comprised of 5 species of trees and 2 species of shrubs. Goats were observed to prefer 10 species (45.45%) which consisted of 6 species of trees and 4 species of shrubs. Sheep grazed on 10 species (45.45%) which were composed of 6 species of trees and 4 species of shrubs. Whereas, 20 species (90.91%) were preferred by camel which consisting of 7 species of trees and 13 species of shrubs (*Table 4*).

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Table 4. *Palatability classification of browse species in Cholistan rangelands*

S.N.	Plant Species	Degree of palatability				Palatability by parts used				Palatability by livestock			
		Hp	Mp	Lp	Np	Lv	Sh	Fl	Fr	Ca	Go	Sh	Cm
1	*Aerva javanica* (Burm. f.) Merill.	−	−	−	+	−	−	−	−	−	−	−	−
2	*Aerva pseudotomentosa* ssp. bovei. Clarke.	−	−	−	+	−	−	−	−	−	−	−	−
3	*Calotropis procera* (Aiton.) Aiton.	−	−	+	−	+	−	−	−	+	+	+	−
4	*Leptadenia pyrotecnica* (Forssakal.) Decne.	−	−	−	+	−	−	−	−	−	−	−	−
5	*Capparis decidua* (Forsskal.) Edgew.	−	+	−	−	−	+	−	−	−	−	−	+
6	*Capparis spinosa* Linn.	−	+	−	−	−	+	−	−	−	−	−	+
7	*Haloxylon recurvum* Bunge. ex. Boiss.	−	+	−	−	−	+	−	−	−	−	−	+
8	*Haloxylon salicornicum* (Moq.) Bunge.	−	−	+	−	−	+	−	−	−	−	−	+
9	*Salsola baryosma* (Roem. et. Scult.) Dany.	−	+	−	−	−	+	−	−	−	−	−	+
10	*Suaeda fruticosa* (Linn.) Farsskal.	−	+	−	−	+	+	−	−	−	−	−	+
11	*Pulicaria rajputanae* Blatt. & Hall.	−	−	+	−	+	−	−	−	−	−	−	+
12	*Abutilon muticum* (Del. ex. DC.) Sweet.	−	−	+	−	+	−	−	−	−	−	−	+
13	*Acacia jacquemontii* Benth.	+	−	−	−	+	+	+	−	−	+	+	+
14	*Acacia nilotica* (Linn.) Del	+	−	−	−	+	+	+	+	+	+	+	+
15	*Prosopis cineraria* (Linn.) Druce.	+	−	−	−	+	+	+	+	+	+	+	+
16	*Prosopis juliflora* DC.	−	+	−	−	+	−	−	+	−	+	+	+
17	*Crotalaria burhia* Ham. Ex. Bth.	−	−	+	−	+	−	−	−	−	−	−	+
18	*Tephrosia uniflora* Pers.	−	−	+	−	+	−	−	−	−	+	+	−
19	*Calligonum polygonoides* Linn.	−	+	−	−	−	+	+	−	−	−	−	+
20	*Zizyphus mauritiana* Lam.	+	−	−	−	+	−	−	−	+	+	+	+
21	*Zizyphus nummularia* (Burm. f.) Wifht & Arn.	+	−	−	−	+	−	−	−	+	+	+	+
22	*Zizyphus spina christi* (Linn.) Wild.	+	−	−	−	+	−	−	−	+	+	+	+
23	*Salvadora oleoides* Decne.	+	−	−	−	+	+	−	−	+	+	+	+
24	*Tamarix aphylla* (Linn.) Karst.	−	+	−	−	−	+	−	−	−	−	−	+
25	*Tamarix dioica* Roxb.	−	+	−	−	−	+	−	−	−	−	−	+

Key words: Hp-Highly palatable, Mp-Moderately-palatable, Lp-Less palatable, Np-Non-palatable; Lv-Leaf, Sh-Shoot, Fl-Flower, Fr-Fruit; Ca-Cattle, Go-Goat, Sh-Sheep, Cm-Camel

Discussion

Forage production of browses

The present study is endeavoring to assess biomass productivity of browse vegetation in Cholistan rangelands. According to results, total dry matter production during wet season was 8029.10 kg/ha while in dry season was 5422.90 kg/ha, that was 19.38% higher in wet season. Whereas dry matter production at sandunal habitat was 21.14% high, at interdunal habitat was 16.6% high and at clayey saline habitat was 22.62% high in wet season as compare to dry season. Overall forage productivity was high during wet season across the three range habitats; this was perhaps due to high rainfall received

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during wet season as compared to dry season. Annual rainfall in Cholistan desert is extremely unpredictable both on temporal and spatial scales. Rainfall generally occurs during monsoon (July to September) and in winter and spring (January to March). The winter rain is scanty so vegetation in spring is usually poor, characterized by few annual species of forbs and grasses providing low biomass for grazing. In arid and semi-arid ecosystems, rainfall is a major environmental agent, affecting the forage productivity and it is extremely variable round the year (Patton et al., 2007). It was observed that Cholistan rangelands are monsoonal and forage productivity of these rangelands depends greatly on monsoon rain. Numerous studies have revealed that rainfall greatly affects the rangelands production and our conclusions agree with them (Farooq, 2003; Durrani and Hussain, 2005).

The rangelands in arid and semi-arid areas are mainly composed of perennial plants, which make the excellent use of climate and soil (Scasta and Rector, 2014). Based on results, it was observed that in both seasons dry forage production was high at interdunal habitat followed by sandunal and clayey saline habitats. This might be due to high vegetation diversity and better water retention capacity of soil at interdunal habitat. Vegetation coverage was low on sand dunes and unstable sand dunes were lacking vegetation. Whereas in clayey saline habitat soil and vegetation structure was poor, might be due to high pH. Being very saline and impermeable to water the clayey saline habitat remained predominately plantless. It was observed that poor soil with less water holding capacity and low nutrients in Cholistan decrease the vigor and size of plants leading to reduced biomass (Katjiua and Ward, 2007).

Generally, difference in productivity level at selected range sites was due to variations in soil, vegetation type, and grazing pressure. Results showed that range sites with high forage productivity were less disturbed by grazing while sites with minimum forage productivity were mostly overgrazed. Both overgrazing and under grazing have adverse effects but overgrazing is more problematical (Gamoun, 2014). During wet season, maximum sites were moderately grazed while in dry season, maximum sites were overgrazed. There was no site without grazing in both seasons. In Cholistan rangelands, grazing period starts from August until February in good rainy years. About all forages were exploited during monsoon and post monsoon season, whereas some green browse remained available throughout year. The commencement of monsoon rains mostly commands the movement in nomadic peoples and livestock. In month of March or April, shortage of water and feed resources in interior of desert compel nomadic peoples and their herds to move towards irrigated plains (Akhtar and Arshad, 2006).

Range carrying capacity of browses

According to results overall browse productively was low in Cholistan rangelands whereas production was high in wet season as compare to dry season. As grazing animals in Cholistan rangelands comprised of cattle, sheep, goats, and camels therefore, carrying capacity was calculated for these kinds of animals. Holechek (1988) has determined daily dry matter (DM) intake for bighorn sheep, elk, moose, white-tailed deer, mule deer, and pronghorn antelopes as two percent of their body weight. As DM intake for the livestock of Cholistan rangelands has not been analyzed yet, thus DM intake for these animals was also taken as two percent of their live-body weight. Based on evidences of livestock producers of the area, a young cow (equal to one AU) may attain average live-body weight of about 350 kg whereas DM requirement of an AU was calculated as 7 kg ha^{-1}.

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Grazing time depends on availability, accessibility, and quality of feed which is reduced when quality forage is abundant (Khumalo et al., 2007). Based on USA recommendations, range utilization intensity is 30 to 40% of key species with 130 to 300 mm annual rainfall for shrub steppe in semiarid region. It may reach at 50% utilization level during high productive year and decrease during dry period (Holechek, 1988). In arid rangelands of Cholistan, range use intensity was taken as 40%. Overall, available browse production during wet season was 157.97 kg/ha and 105.88 kg/ha during dry season. Based on these standards; overall carrying capacity during wet season was 16 ha/AU/Y and in dry season was 24 ha/AU/Y. Whereas on browse forage availability, in wet season 80376 AU/Y while in dry season 53872 AU/Y were estimated that can be grazed in Cholistan rangelands.

Based on three range habitats in Cholistan, carrying capacity was high at interdunal habitat followed by sandunal and clayey saline habitats during the both seasons. Forage production was better in wet season than dry season (April to June) therefore, carrying capacity was high in wet season as compare to dry season. The data suggested that carrying capacity during both seasons was very low and available browse production was insufficient for present stocking rate (Guevara et al., 2009).

Similarly, nomadic peoples in Cholistan desert have also exploited the plant resources of this area. They uprooted almost every plant for their need irrespective of its forage, medicinal or other values. Therefore, forage productivity is decreasing day by day ultimately leading to poor carrying capacity. No doubt these rangelands are dominated by non-equilibrium condition. In this environment, drought will always be a key factor in affecting production level and animal numbers. Whereas, to decide grazing prescriptions a resource manager needs to define variations in productivity levels and provide estimates of stocking rates (Bisigato et al., 2005).

Palatability classification of browses

It was observed that identified browse species comprising 7 species of trees and 18 species of shrubs were almost remained available throughout the year in Cholistan rangelands. As rangelands vegetation varied significantly in their seasonal availability, nutritive value, and palatability, so grazing animals apparently select highly palatable forage species first (Heitschmidt et al., 2005). According to results, maximum browse species were found to be moderately palatable. However, seven species were highly palatable which consist of 5 species of trees (*Acacia nilotica, Prosopis cineraria, Zizyphus mauritiana, Zizyphus spina Christi, Salvadora oleoides)* and 2 species of shrubs (*Acacia jacquemontii, Zizyphus nummularia)*. Whereas, three species were found to be unpalatable which consist of only shrubs including *Aerva javanica, Aerva pseudotomentosa* and *Leptadenia pyrotechnica*. This unpalatability might be due to alkaloids, phenolics, saponins and other poisonous elements. It is very difficult to differentiate between non-poisonous and poisonous plants as animal's dislike feed due to unlikable feelings or physical discomfort, or by excess or deficiency of nutrients (Kayani et al., 2007).

Animals greatly differ in their preferences for selection of various plant species or plant parts as feed. Results showed that maximum trees were used for their leaves, whereas maximum shrubs were preferred for their shoot. Leaves have been grazed in maximum browse species (63.64%), as feed by grazing animals. The livestock usually prefer leaves of all forages, might be due to high crude protein, phosphorus and low lignin and fiber contents than woody parts. Generally, animals desire fresh foliages than

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dried and non-succulent forages that can be eaten easily. Likewise, soft green herbaceous parts, in addition having good taste and odour are rapidly digestible (Sanon et al., 2007). It was also observed that *Acacia nilotica, Prosopis cineraria,* and *Prosopis juliflora* were preferred for their fruits by livestock. Flowers and fruits are seasonally essential in animal feed as they might have high level of proteins and cell soluble than leaves. Fresh forage species with high contents of crude protein, sugar, cellulose and fats are highly preferred and digestible. While plant species with high lignin, fiber, silica, secondary metabolites and with poor digestibility are less preferred by grazing animals (Holechek et al., 1998).

Rangelands of Cholistan desert are freely grazed by mixed herds of cattle, sheep, goats and camels. According to results, camel ranked first in exploring maximum number of species, which consist of 7 species of trees, 13 species of shrubs. Goat and sheep were observed to have similar selection, which consisted of 6 species of trees and 4 species of shrubs. Cattle were observed to utilize minimum number of species including 5 species of trees and 2 species of shrubs. It was observed that grazing animals select the most palatable plant species first. It may lead to complete replacement of good quality forages by non-palatable species (Rutherford and Powrie, 2013). Animals face forage deficiency in Cholistan rangeland in winter (December and January) but this condition become severe in April owing to climatic extremities and shortage of water. Certain perennial browse species continue to maintain their foliage but deficiency of forage forces the animals to eat even less palatable species. It may be possible that poor health of animals in Cholistan rangelands are partially due to continuous utilization of such unpalatable species (Deng et al., 2013).

This study revealed that area was vegetative rich in wet season as compare to dry season. Browse species were found playing significant role in the provision of forage for livestock round the year particularly during droughts. The data suggested that carrying capacity during both seasons was very low and available browse production was insufficient for present stocking rate. Due to continuous grazing pressure, the palatable species were disappearing and unpalatable species were spreading on the landscape. It was observed that in Cholistan rangelands maximum forage was available during monsoon season because sustainability of life in this desert rotates round the annual precipitation. Numerous species of ephemeral and annual appear after rains, complete their life cycle in a short duration and vanish. These species, as well important in nutritional contribution also decrease grazing pressure on palatable perennial browse species (Gamoun et al., 2015).

Conclusions

This study has provided baseline about seasonal forage productivity, carrying capacity and palatability of browse vegetation in Cholistan rangelands. However, this study is very preliminary and it is recommended that subsequent ecological studies should be conducted on spatial and temporal variations about forage production. The productive potential of rangelands is not constant and carrying capacities need to be periodically reviewed to accommodate any changes in land resources, or environment. There is severe problem of overgrazing that leads to year-round stress on browse species. Grazing at suitable stocking rate is compulsory. Planned grazing should be introduced and implemented to release the stress over browse species. All factors considered, it was concluded that Cholistan rangelands are less productive and they

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need proper protection, management, and rehabilitation through ecological approaches. This data should be incorporated into the current management plan and subsequent vegetation map should serve as a valuable tool in planning, conservation and management of these rangelands.

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GENETIC DIVERSITY AND POPULATION STRUCTURE AMONG 98 MAIZE INBRED LINES INVESTIGATED WITH SSR MARKERS

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Abstract. The conversion of exotic germplasm into domestic maize breeding materials is essential to solve the narrow genetic base for maize improvement in China. The present study applied SSR markers and three complementary cluster methods (STRUCTURE, UPGMA and PCoA) to 98 foreign hybrid-selected lines for effective hybrid breeding. A total of 450 alleles, with an average of 3.98 alleles per locus, were detected**.** Among 10 chromosomes, there were 13-19 loci and 33-59 alleles with averaged alleles 2.54~3.43 per chromosome. The polymorphism information content (PIC) among 10 chromosomes ranged from 0.4504~0.5582 and 69.4% PIC variation were explained by allele number/Locus. The highest PIC was observed in chromosome 7 (0.5582) and the lowest in chromosome 8 (0.4965). The STRUCTURE clustering analysis grouped the test lines into four subpopulations (i.e. REID, Lancaster, P and Domestic) in accordance with UPGMA and PCoA clustering**.** The higher genetic diversity were detected among the inbred lines in P and Domestic subpopulations. The allele frequencies, gene diversity and population structure obtained in the present study lead us to conclude that the 98 inbred lines derived from foreign hybrid-selected lines contain extensive genetic variation and are a valuable resource for Chinese maize breeding. The result obtained in the present study will assist in effective utilization of the lines in Chinese hybrid maize breeding programs.
Keywords: *maize germplasm, hybrid-selected line, genetic variation, clustering analysis, maize breeding*

Introduction

Knowledge of genetic diversity and population structure among inbred lines and breeding materials is of great importance for maize hybrid breeding. With the popularization and application of maize hybrids over the past years, the recurrent use of a few elite germplasm lines as parental stock has led to a decrease in genetic diversity among maize breeding materials in China. The introduction of exotic germplasm, using its abundant genetic variation and good agronomic traits, is therefore essential to solve the narrow genetic base for maize improvement in China (Wen et al., 2012; Yong et al.,

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2013). However, it is necessary to make a comprehensive evaluation on the genetic diversity and population structure of exotic germplasm (Tarter et al., 2004; Šarčević et al., 2008; Živanović et al., 2012).

Molecular markers can be employed to investigate levels of genetic diversity and population structure among maize inbred lines and breeding materials. SSRs, due to its abundant, highly polymorphic, genome specific, codominant in nature, have found application in analyses of genetic diversity, population structure, gene mapping, and assisted selection for maize improvement (Phumichai et al., 2012; Wende et al., 2013; Semagn et al., 2014; Yang et al., 2013; Abakemal et al., 2015).

In the present study, 98 foreign hybrid-selected lines were analyzed using 145 SSR loci distributed over the whole maize genome. Our objectives were to estimate the levels of genetic diversity and population structure. The results will be useful to breeders in selecting the best parental combinations for maize breeding program in China.

Research Design and Methods

Plant Materials

The germplasm contained 5 tester lines (Huangzao 4, Dan 340, B73, Qi 319 and Mo17) and 98 maize inbred lines derived from foreign maize hybrid-selected lines (obtained by Liaoning Leiao seed company, China) was used in this study. The pedigree and/or origin information can be found in *Appendix 1*.

SSR Markers and Genotyping

Genomic DNA was extracted from approximately 200 mg fresh leaf tissue using the cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984). A total of 500 SSR primers, which were distributed evenly over the 10 maize chromosomes, were selected and synthesized according to the information available in the MaizeGDB database (http://archive.maizegdb.org/).

PCR amplifications were carried out in 10 mL reaction volumes containing 1 µL template DNA, 2 µL each of 2.5 mM primer, 5µL 2×Taq Master Mix, 0.1µL of 5 units μL^{-1} Taq DNA polymerase, 0.4µL 10 mM dNTPs and , dH2O 2µL. PCR protocols consisted of 32 cycles of 94℃ for 45s, an annealing temperature at either 45, 50, 55 or 60°C depending on the individual SSR primers for 45 s, and 72°C for 60s, and a final extension step of 72°C for 10 min. PCR products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining.

Genetic Diversity Analysis

For each SSR locus, polymorphic bands were scored as 1 or 0 for presence or absence of the bands at the same mobility, respectively. Gene diversity (PIC) was calculated for each marker according to the formula: PIC=1－∑fi2, where fi is the allele frequency for the i-th locus summed across all alleles for that locus. The program PowerMarker v3.25 and Excel was used to calculate allele number, allele frequency, and gene diversity of each locus (Liu et al., 2005).

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Population Structure Analysis

The STRUCTURE v2.3.3 were employed to assess the population structure of the 98 maize inbred lines using the Bayesian model-based approach (Pritchard et al., 2000). The number of subgroups (K), with each K repeated five times, was ranged from 1 to 12, with burn-in of 100,000 and run length of 100,000. We used the ad hoc criterion ΔK related to the second order rate of change in the log probability of data (LnP(D)) to determine the most probable K value (Evanno et al., 2005).

To examine genetic relationships among the 98 maize inbred lines, the data matrices of the genetic similarity were used to create the dendrogram using UPGMA clustering with the computer software NTSYS-pc v2.2 (Rohlf 2009). Principal coordinate analysis (PCoA) was also employed to reveal relationships among the 98 inbred lines using the software JMPversion7.0 (SAS Institute Inc., Cary, NC, USA).

Data Analysis and Results

A total of 500 SSRs, randomly distributed across the maize genome, were used to evaluate the genetic diversity of the 98 maize inbred lines. Finally, 145 SSRs with clear, stable and specific bands were selected to scored on the 98 lines, with an average of 3.98 alleles per locus (range of 2-7). The PIC for all loci ranged from 0.2130 (umc1271) to 0.8316 (bnlg1666) with an average value of 0.5067 (*Appendix 2*). The higher PIC values indicated the high variability of SSRs, and also a large genetic difference among the 98 maize inbred lines.

The SSRs among 10 maize chromosomes ranged from 11 to 19 with allele number from 33-59 *(Table 1)*. The highest allele number was detected on chromosome 1 (59 alleles), followed chromosome 6 (51 alleles), and the lowest on chromosome 8 (33 alleles). For all chromosomes, there were 13-19 loci and 33-59 alleles with averaged alleles 2.54~3.43 per chromosome. The PIC among 10 chromosomes ranged from 0.4504~0.5582. The highest PIC was observed in chromosome 7 (0.5582) and the lowest in chromosome 8 (0.4965).

Table 1. *Genetic diversity at genome level of 98 maize inbred lines revealed by 145 SSR markers*

Chrom	No. of Loci	No. of Alleles	Mean allele number (range)	PIC
1	19	59	3.11	0.4897
2	13	43	3.31	0.5336
3	14	47	3.36	0.5532
4	17	51	3.00	0.5199
5	16	49	3.06	0.5036
6	16	51	3.19	0.4958
7	14	48	3.43	0.5582
8	13	33	2.54	0.4504
9	12	35	2.92	0.4507
10	11	34	3.09	0.5075

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There usually was a positive linear relationship between the polymorphism information content (PIC) and number of alleles within a given range. Simple correlation analysis indicated that the PIC was significantly and positively correlated with the number of alleles (r=0.6566, p<0.001) (*Fig. 1*). The allele number/Locus (x) could explain 69.4% PIC variation (y) estimated by a curvilinear regression equation (y =0.3286 ln (x) +0.1551 (1<x<8), R^2 =0.6937).

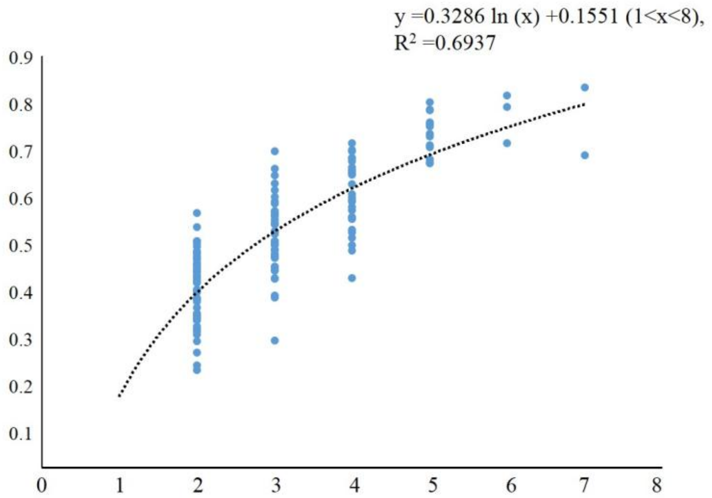

Figure 1. *Plot of PIC vs. number of alleles per SSR locus Population Structure Analysis {tc "5.2.3 Analysis of population structure of mazie core collection" \l 000003}*

STRUCTURE V2.3.3 software was employed to assess the population structure of 98 inbred lines based on 145 whole-genome unlinked SSR markers. LnP(D) progressively increased as K (2-10) increased and no obvious inflexion point was observed, which LnP(D), in this case, may not be suitable to estimate the true K value (*Fig. 2*). The peak of ΔK was observed at K=4, suggested that the 98 inbred lines were fell into four sub-populations (*Fig. 2*).

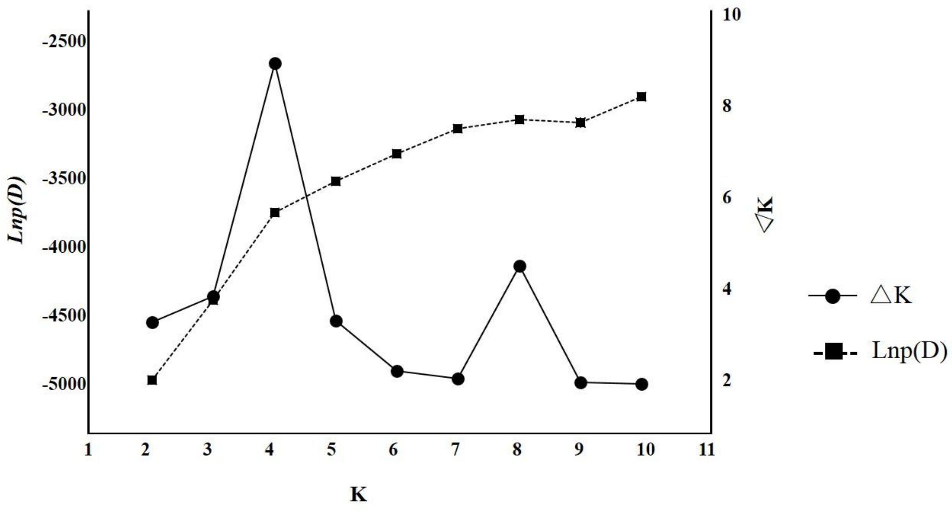

Figure 2. *Plot of LnP (D) and △K calculated for K ranging from 1 to 10*

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Based on the maximum membership probability, 98 inbred lines were assigned into 4 subpopulations (Reid, Lancaster, P, Domestic) (*Fig.3*). Reid included 35 inbred lines closed to B73 (Reid) genetic background. Lancaster had 26 inbred lines related to Mo17 (Lancaster) genetic background. P comprised 22 inbred lines, most derived from Pioneer hybrids (P). The other 15 inbred lines were closed to Dan 340 or Huangzao 4 background, which named as Domestic subgroup described by Xie et al. (2008).

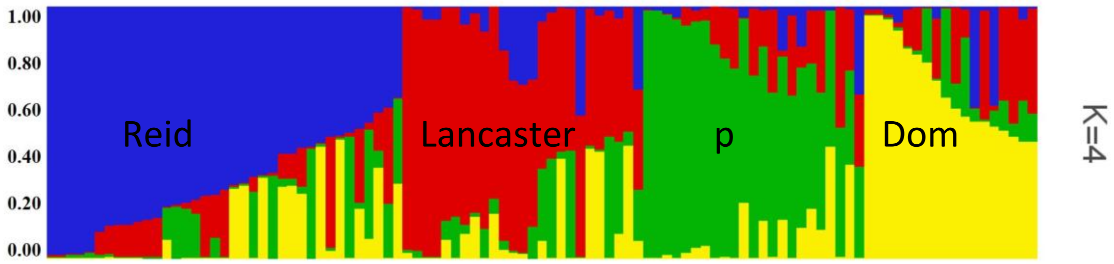

Figure 3. *Population structure of the 98 inbred lines (K=4). Note: The horizontal coordinate represents the 98 inbred lines, and the vertical coordinate of each subgroup indicates the membership coefficients for each individual. Blue zone: Reid; Red zone: Lancaster; Green zone: P; Yellow zone: Domestic*

The similarity coefficient among 98 maize inbred lines ranged from 0.2692 to 0.9825 and the average was 0.6564. When the similarity coefficient was 0.57, UPGMA cluster analysis also clearly grouped 98 inbred lines into four subpopulations (*Fig.4*). Reid, Lancaster P and Domestic subpopulation comprised 36, 32, 13 and 17 inbred lines, respectively. The assignments of 77 inbred lines (78.6% of the total) by UPGMA clustering were consistent with their assignments using STRUCTURE.

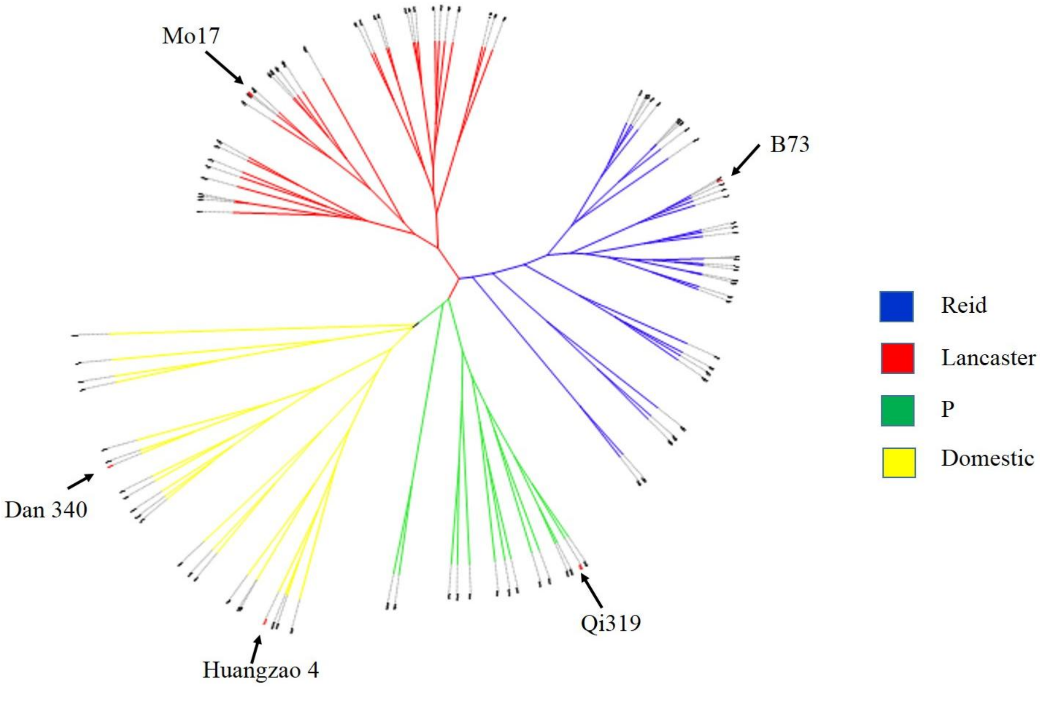

Figure 4. *Dendrogram of 98 maize inbred lines based on UPGMA cluster analysis*

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The principal coordinate analysis (PCoA) based on 145 SSR markers also separated the 98 maize inbred lines into four major groups (*Fig.5*). As inferred by STRUCTURE analysis, the inbred lines in Reid were mainly distributed in the lower left of the plot resulting, Lancaster distributed in the upper right, P in the upper left, and Domestic in the upper right. Most individuals within Reid and Lancaster subpopulations were grouped more closely. The inbred lines in P and Domestic were widely scattered, indicating that higher genetic diversity resided in P and Domestic subpopulations.

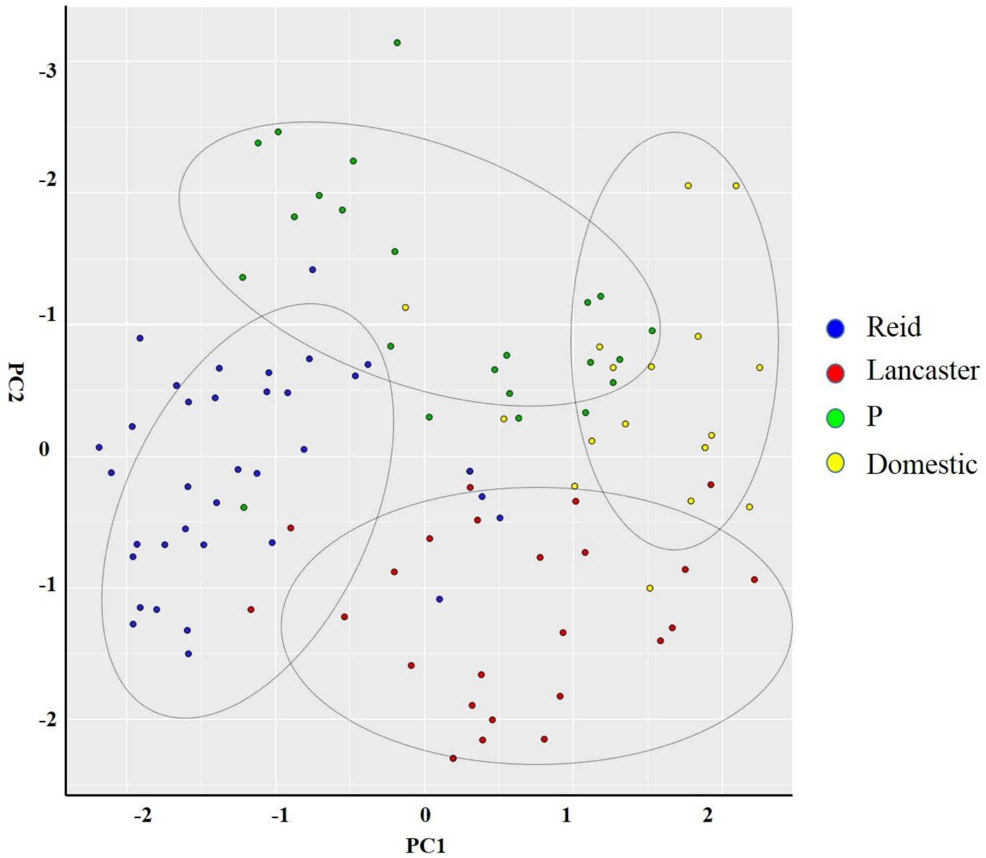

Figure 5. *Principal coordinate analysis of 98 maize inbred lines based on 145 SSR markers. Reid (blue), Lancaster (red), P (green) and Domestic (yellow) are the four subgroups identified by STRUCTURE assigned with the maximum membership probability*

Discussion and Conclusion

SSR markers, due to their abundance, co-dominance, and locus specificity, have been extensively used to assess genetic diversity in maize genotypes (Šarčević et al., 2008; Inghelandt et al., 2010).

In the present study, all the 98 tested maize inbred lines were derived from foreign hybrid-selected lines. We used 145 SSR markers to screen the population and a total of 450 alleles, with an average of 3.103, were detected. The average polymorphic information content (PIC) value was 0.5067, which was lower than that in Chinese important inbred lines with PIC over 0.6 (Wang et al., 2008; Xie et al., 2008). The number of alleles found in this study is also in agreement with other studies (Wang et al., 2008; Park et al., 2015). Wang et al. (2008) reported a total of 1,365 alleles with an

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average of 9.4 alleles per locus by screening 95 inbred lines using SSR markers. Park et al. (2015) genotyped 174 maize inbred lines by 150 SSR markers and detected a total of 1082 alleles with an average of 7.21 alleles per locus. In our study, alleles were obtained at the whole genome level (*Table 1*). Chromosome 1 showed the highest allele number (59 alleles) and chromosome 8 the lowest (33 alleles). Therefore, we have thus determined that there is a higher level genetic diversity in the 98 foreign hybrid-selected lines, which has the potential to enhance the genetic diversity of Chinese maize breeding materials.

Population structure in the present study was also investigated using three complementary analysis methods STRUCTURE, UPGMA and PCoA based on SSR data. We selected maximum membership probability as the subgroup subdivision criterion, 98 maize inbred lines were assigned into four subpopulations which was in agreement with the assignments obtained by UPGMA and PCoA clustering. Nevertheless, for the 98 tested foreign hybrid-selected lines, the pedigree information was not in accordance with their clustering. In our data, the Domestic subgroup closed to Dan 340 or Huangzao 4 background. This finding can be partially explained by complex genetic background in foreign hybrids. Therefore, it is of significant importance to understand population structure and relationships among inbred lines is for maize improvement.

The allele frequencies, gene diversity and population structure obtained in the present study lead us to conclude that the 98 inbred lines derived from foreign hybrid-selected lines contain extensive genetic variation and are a valuable resource for Chinese maize breeding.

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APPENDIX

Appendix 1. *Germplasm sources and pedigrees of 103 maize acessions*

ID	Inbred line	Pedigree/source
1	Huangzao4	Landrace Tangsipingtou
2	Mo17	C103×187-2
3	Dan340	Baigulu9×Z. mays-tunicata
4	Qi319	selected from Pioneer hybrid ‘‘78599’
5	B73	Iowa Stiff Stalk Synthetic C5 (BSSS)
6	Y005	selected from American hybrid “DK516”
7	Y025	selected from American hybrid “DK516”
8	Y052	selected from American hybrid “DK516”
9	Y057	selected from American hybrid “DK516”
10	Y058	selected from American hybrid “DK516”
11	Y087	selected from American hybrid “DK516”
12	Y088	selected from American hybrid “DK516”
13	Y097	unknown
14	Y099	unknown
15	Y101	unknown
16	Y102	unknown
17	Y113	selected from American hybrid “DK007”
18	Y136	selected from American hybrid “DK007”
19	Y187	selected from American hybrid “DK007”
2	Y195	selected from American hybrid “DK007”
21	Y199	selected from American hybrid “DK007”
22	Y210	selected from American hybrid “DK007”
23	Y219	selected from American hybrid “DK008”
24	Y220	selected from American hybrid “DK008”
25	Y255	selected from American hybrid “DK008”
26	Y263	selected from American hybrid “DK008”
27	Y275	selected from American hybrid “DK008”
28	Y282	selected from American hybrid “DK008”
29	Y295	selected from American hybrid “DK008”
30	Y302	selected from an American hybrid
31	Y316	selected from an American hybrid
32	Y323	selected from an American hybrid
33	Y354	selected from American hybrid “3425”
34	Y361	selected from American hybrid “3425”
35	Y362	selected from American hybrid “3425”
36	Y386	selected from American hybrid “3425”
37	Y393	selected from Pioneer hybrid ‘‘XY335”
38	Y403	selected from Pioneer hybrid ‘‘XY335”
39	Y415	selected from Pioneer hybrid ‘‘XY335”
40	Y416	selected from Pioneer hybrid ‘‘XY335”
41	Y420	selected from Pioneer hybrid ‘‘XY335”
42	Y421	selected from Pioneer hybrid ‘‘XY335”
43	Y426	selected from Pioneer hybrid ‘‘XY335”
44	Y431	selected from Pioneer hybrid ‘‘XY335”
45	Y437	selected from Pioneer hybrid ‘‘XY335”
46	Y443	selected from Pioneer hybrid ‘‘XY335”
47	Y464	selected from Pioneer hybrid ‘‘XY335”

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48	Y467	selected from Pioneer hybrid ‘‘XY335”
49	Y505	selected from Pioneer hybrid ‘‘PR3394”
50	Y509	selected from Pioneer hybrid ‘‘PR3394”
51	Y525	selected from Pioneer hybrid ‘‘PR3394”
52	Y531	selected from Pioneer hybrid ‘‘PR3394”
53	Y532	selected from Pioneer hybrid ‘‘PR3394”
54	Y569	unknown
55	Y570	unknown
56	Y594	selected from American hybrid‘‘3382”
57	Y625	selected from American hybrid ‘‘3382”
58	Y645	selected from American hybrid ‘‘3382”
59	Y647	selected from American hybrid ‘‘3382”
60	Y648	selected from American hybrid ‘‘3382”
61	Y649	selected from American hybrid ‘‘3382”
62	Y683	selected from Pioneer hybrid ‘‘XY222”
63	Y700	selected from Pioneer hybrid ‘‘XY222”
64	Y721	selected from Pioneer hybrid ‘‘XY222”
65	Y728	selected from Pioneer hybrid ‘‘XY222”
66	Y733	unknown
67	Y751	unknown
68	Y753	unknown
69	Y760	selected from Pioneer hybrid ‘‘78599’
70	Y764	selected from Pioneer hybrid ‘‘78599’
71	Y769	selected from Pioneer hybrid ‘‘78599’
72	Y777	selected from Pioneer hybrid ‘‘78599’
73	Y793	selected from Pioneer hybrid ‘‘78599’
74	Y815	selected from Pioneer hybrid ‘‘78599’
75	Y829	selected from Pioneer hybrid ‘‘XY508”
76	Y837	selected from Pioneer hybrid ‘‘XY508”
77	Y839	selected from Pioneer hybrid ‘‘XY508”
78	Y851	selected from Pioneer hybrid ‘‘XY508”
79	Y882	selected from Pioneer hybrid ‘‘XY508”
80	Y886	selected from Pioneer hybrid ‘‘XY508”
81	Y889	selected from Pioneer hybrid ‘‘XY508”
82	Y895	selected from Pioneer hybrid ‘‘XY508”
83	Y897	selected from Pioneer hybrid ‘‘XY508”
84	Y918	selected from Pioneer hybrid ‘‘33G35”
85	Y919	selected from Pioneer hybrid ‘‘33G35”
86	Y920	selected from Pioneer hybrid ‘‘33G35”
87	Y963	selected from Pioneer hybrid ‘‘33G35”
88	Y966	selected from Pioneer hybrid ‘‘33G35”
89	Y1026	selected from Pioneer hybrid ‘‘33G35”
90	Y1043	selected from Pioneer hybrid ‘‘32T24”
91	Y1045	selected from Pioneer hybrid ‘‘32T24”
92	Y1052	unknown
93	Y1069	unknown
94	Y1071	unknown
95	Y1081	unknown
96	Y1084	selected from Pioneer hybrid ‘‘33F20”
97	Y1092	selected from Pioneer hybrid ‘‘33F20”
98	Y1094	selected from Pioneer hybrid ‘‘32D22”
99	Y1099	selected from Pioneer hybrid ‘‘32D22”
100	Y1115	selected from Pioneer hybrid ‘‘32D22”

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101	Y1124	selected from Pioneer hybrid ‘‘32D22”
102	Y1161	selected from Pioneer hybrid ‘‘32D22”
103	Y1183	selected from Pioneer hybrid ‘‘32D22”

Appendix 2. *Chromosome locations for 145 SSRs, and their allele numbers and PIC value detected in 98 inbred lines of maize*

Primer Name	Bin	No. of alleles	PIC	Primer Name	Bin	No. of alleles	PIC
umc985	1.06	6	0.7085	umc2400	5.04	3	0.4399
umc1166	1.02	2	0.4301	bnlg389	5.09	4	0.5027
umc1243	1.04	2	0.3888	bnlg1046	5.03	3	0.4637
umc1292	1.01	3	0.4136	bnlg1118	5.07	3	0.4752
umc1358	1.07	2	0.3291	bnlg1237	5.05	5	0.6998
umc1383	1.08	2	0.3082	bnlg2323	5.04	2	0.3004
umc1568	1.02	3	0.5166	umc1250	6.05	4	0.4149
umc1590	1.06	4	0.5438	umc1296	6.06	2	0.3681
umc1706	1.07	3	0.5156	umc1413	6.05	4	0.6408
umc1744	1.11	2	0.3860	umc1474	6.05	2	0.2240
umc1972	1.06	2	0.3636	umc1490	6.07	2	0.3697
umc2012	1.01	4	0.6776	umc1653	6.07	5	0.7820
umc2025	1.05	3	0.4921	umc1656	6.02	3	0.4869
umc2240	1.08	2	0.3318	umc1887	6.03	2	0.4445
bnlg439	1.03	4	0.6564	umc1918	6.04	3	0.5316
bnlg1014	1.01	3	0.4587	umc2006	6.04	3	0.4398
bnlg1025	1.07	4	0.5626	umc2059	6.08	2	0.4585
bnlg1866	1.03	6	0.8137	umc2165	6.07	4	0.5976
bnlg2238	1.04	2	0.4073	umc2312	6.01	3	0.3758
umc1026	2.04	2	0.4426	bnlg107	6.01	4	0.5818
umc1265	2.02	2	0.3364	bnlg161	6.00	5	0.7235
umc1419	2.00	3	0.4313	bnlg1538	6.01	3	0.4943
umc1755	2.06	3	0.5509	umc1066	7.01	2	0.4273
umc1845	2.03	3	0.5324	umc1159	7.01	4	0.6501
umc1875	2.06	4	0.6769	umc1213	7.02	2	0.4040
umc2094	2.01	5	0.7983	umc1407	7.05	3	0.6391
umc2129	2.07	4	0.6692	umc1593	7.03	7	0.6825
umc2214	2.10	2	0.3260	umc1642	7.00	3	0.6911
umc2372	2.06	4	0.5199	umc1718	7.03	2	0.2511
bnlg1017	2.02	3	0.4619	umc1782	7.04	5	0.7452
bnlg1258	2.08	5	0.7293	umc1799	7.06	4	0.6917
bnlg1520	2.09	3	0.4612	umc1944	7.04	2	0.4672
umc1501	3.05	2	0.3027	umc1983	7.02	2	0.4719
umc1539	3.05	4	0.6191	umc2057	7.07	3	0.4930
umc1886	3.02	3	0.5347	bnlg1666	7.04	7	0.8316

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umc2049	3.01	2	0.3834	bnlg2132	7.00	2	0.3683
umc2118	3.00	2	0.4951	umc1130	8.05	2	0.4149
umc2263	3.04	3	0.4672	umc1139	8.01	2	0.3220
umc2266	3.06	2	0.4086	umc1161	8.06	2	0.4546
umc2273	3.07	3	0.5603	umc1384	8.07	3	0.5105
umc2369	3.03	2	0.4614	umc1530	8.03	3	0.4855
bnlg197	3.06	5	0.6653	umc1638	8.09	2	0.5568
bnlg1350	3.08	4	0.6432	umc1663	8.09	3	0.4956
bnlg1496	3.09	5	0.6669	umc1728	8.06	2	0.2907
bnlg1754	3.09	5	0.7816	umc1846	8.05	4	0.4745
bnlg2241	3.06	5	0.7553	umc1984	8.03	2	0.4445
umc1017	4.01	3	0.5118	bnlg666	8.05	4	0.5909
umc1132	4.08	3	0.6059	bnlg1863	8.03	2	0.4831
umc1294	4.02	2	0.3234	bnlg2046	8.04	2	0.3315
umc1716	4.11	3	0.5778	umc1107	9.04	3	0.5137
umc1738	4.10	2	0.4949	umc1271	9.03	2	0.2130
umc1821	4.04	2	0.4281	umc1277	9.07	2	0.2976
umc1847	4.07	2	0.2759	umc1366	9.06	3	0.3711
umc1943	4.02	3	0.4133	umc1492	9.04	2	0.4905
umc2027	4.06	3	0.5921	umc1505	9.08	2	0.3351
umc2039	4.03	2	0.4409	umc1570	9.04	2	0.3240
umc2046	4.09	3	0.5153	umc2128	9.03	3	0.5502
umc2188	4.08	2	0.5254	umc2337	9.03	3	0.4308
bnlg1265	4.05	3	0.4761	umc2343	9.05	5	0.7501
bnlg1444	4.08	5	0.7055	bnlg1191	9.07	4	0.4862
bnlg1621	4.06	4	0.5840	bnlg1401	9.02	4	0.6455
bnlg1890	4.11	4	0.6944	umc1196	10.07	3	0.5811
bnlg2162	4.08	5	0.6742	umc1319	10.01	3	0.5145
umc1019	5.06	6	0.7887	umc1380	10.00	2	0.4580
umc1072	5.07	3	0.6536	umc1477	10.05	4	0.5459
umc1389	5.03	2	0.3367	umc1506	10.05	3	0.5425
umc1478	5.01	2	0.4445	umc1873	10.04	2	0.4724
umc1624	5.04	2	0.3501	umc1877	10.07	3	0.2772
umc1692	5.03	2	0.4182	umc2069	10.02	4	0.5141
umc2036	5.01	3	0.5573	umc2350	10.04	2	0.4190
umc2136	5.08	3	0.6215	bnlg987	10.03	4	0.5482
umc2216	5.06	4	0.5698	bnlg2190	10.06	4	0.7093
umc2386	5.05	2	0.4352				

Note: Bin numbers were determined from maizeGDB

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PREDOMINANCE OF BLOW FLIES (DIPTERA: CALLIPHORIDAE) AMONG INSECTS VISITING FLOWERS OF *BUCHANANIA LANZAN* (SAPINDALES: ANACARDIACEAE)

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Abstract. This study highlighted blow flies as new possible pollinators of *Buchanania lanzan* (Sapindales: Anacardiaceae) flowers. Two sets of 2-day sampling were done by using a sweep net on the flowers of *B. lanzan* in a deciduous forested area of Mahidol University, Nakhonsawan Campus, Nakhon Sawan Province, Thailand during 27-28 December 2013 and 13-14 December 2014. The number of flies (Diptera) collected was larger than the combined number of bees (Hymenoptera) and beetles (Coleoptera) by a factor of 9.4. Blow flies (Diptera: Calliphoridae) were the most abundant insects caught (34.8% of total), and included the medically-important *Chrysomya megacephala. Stomorhina discolor* predominated (23.6% of total), followed by *C. megacephala*, *C. rufifacies*, *C. nigripes* and *Rhyncomya flavibasis*. Female flies predominated over males and were mostly collected in the morning. Many pollen grains were attached to the bodies of the flies, especially on the legs, thorax, and/or abdomen, indicating the likely importance of blow flies as pollinators of the blossoms of *B. lanzan.* These new findings could be useful for furthering conservation management and economic production of *B. lanzan,* and also for the use of *B. lanzan* flowers or the chemicals which they extrude as bait for controlling medically-important fly populations.
Keywords: *Almondette, insect diversity, pollinator, Stomorhina discolor, wasp-mimicking flies*

Introduction

Buchanania lanzan (Spreng) (common name Almondette, family Anacardiaceae) is found growing naturally as a wild plant in subtropical and tropical deciduous forests in many countries, including Thailand (Wongpakam et al., 2007; Sereesongsaeng and Khumchompoo, 2009), Myanmar, Malaysia, India (Martin et al., 1988), Australia and the Pacific islands (Chauhan et al., 2012). The tree is moderate-sized with alternate and simple leaves. Many parts of *B. lanzan* have socio-economic or medicinal importance, particularly in India. Its kernel is a commercially edible nut and is regarded as substitute for almonds in desert habitats (Rosengarten, 1984). Prasad and Bhatnagar (1993) reported that more than one thousand tons of kernels were harvested yearly from native forests of Mahdya Pradesh and Chhattisgarh in India (Duke, 2000). The other parts of this plant are of phytomedical and pharmaceutical importance and are used in Indian folk medicine to treat wounds, skin diseases, and diarrhoea (Patsnaik et al., 2011; Kumar et al., 2012). The bark may also be beneficial for application under conditions of immune stimulation and/or bacterial infection (Sekhar et al., 2015). Unfortunately, no commercial farming of *B. lanzan* has been carried out. Thus, ecological management may be needed to balance the demands of medicinal utility and tree conservation (Chauhan et al., 2012).

During reproduction, the flowers are formed in a panicle. As in the mango, *Mangifera indica*, both staminate flowers and hermaphrodite flowers are present in each panicle, but pollination is achieved by neither self-propagation nor wind pollination.

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Insect pollination is therefore presumed to play a significant role in propagation (Huda et al., 2015).

Blow flies (Diptera: Calliphoridae) are considered to be of medical and veterinary concern, and to have negative impacts on public health worldwide (Greenberg, 1973). From an ecological and agricultural standpoint, however, blow flies are potentially important pollinators of flowers including mango, from which they gather pollen or nectar on their legs and body surfaces during feeding (Anderson et al., 1982; Hu et al., 1995).

At Mahidol University Nakhonsawan Campus, when *B. lanzan* trees were blooming during December 2012, casual visitors to the vicinity of the trees assumed that the buzzing sound was from bees or wasps. After conducting preliminary observations, however, first author found that almost all of insects at the tree were flies such as *Chrysomya megacephala*, *Chrysomya rufifacies* and *Stomorhina discolor*. The objective of this study was therefore to determine the relationship between blow flies and flowers of *B. lanzan.*

Materials and Methods

Study site

Insect sampling was conducted at a *B. lanzan* tree on the Mahidol University Nakhonsawan Campus, Nakhon Sawan Province, north-central Thailand for a two-day period during two successive dry seasons (27–28 December 2013 and 13–14 December 2014), coinciding with the flowering period of *B. lanzan*. The *B. lanzan* tree sampled was located outside the administrative offices of the university (15.57912°N, 100.43717°E) (*Figure 1A*).

Shorea obtusa (Wall. ex Blume.) and *Shorea siamensis* (Miq.) have been found commonly near the study site. They are common trees in a disturbed deciduous dipterocarp forest. In a radius of 20 m, an additional 10 trees of *Azadirachta indica* (A. Juss.) (3), *Diospyros rhodocalyx* (Kurz.) (1), *Maerua siamensis* (Kurz) Pax. (3), *Pterocarpus indicus* (Willd.) (2) and *S. obtusa* (1) were found.

Weather data collected at Nakhonsawan Meteorological Station (15.67213°N, 100.12962°E), ~10 km distance from the study site, showed that daytime maximum temperatures reached 33.8 °C and 35.5 °C during December 2013 and 2014, respectively (www.met-sawan.tmd.go.th). Rainfall was c. 2.4 mm during December 2014, while; no rain fell during December 2013.

Insect sampling on B. lanzan flowers

A sweep net was used to catch all insects swarming over the flowers of *B. lanzan* during 3 h in the morning (0900-1200 h) and 3 h in the afternoon (1300-1600 h), in order to examine the pattern of insect visitation. All insects collected were anesthetized in an ethyl acetate killing jar. The insects were sorted to family using the taxonomic key of Triplehorn and Johnson (2005), and then identified to genus and species (where possible) under a stereo microscope (Olympus, Japan). Blow flies and muscid flies were identified using the taxonomic keys of Kurahashi and Bunchu (2011) and Tumrasvin and Shinonaga (1977), respectively. Unknown species were grouped as morpho-species.

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All insects were pinned and observed for attachment of pollen under the stereo microscope. Finally, all insects were deposited in preservation boxes at Mahidol University Nakhonsawan Campus.

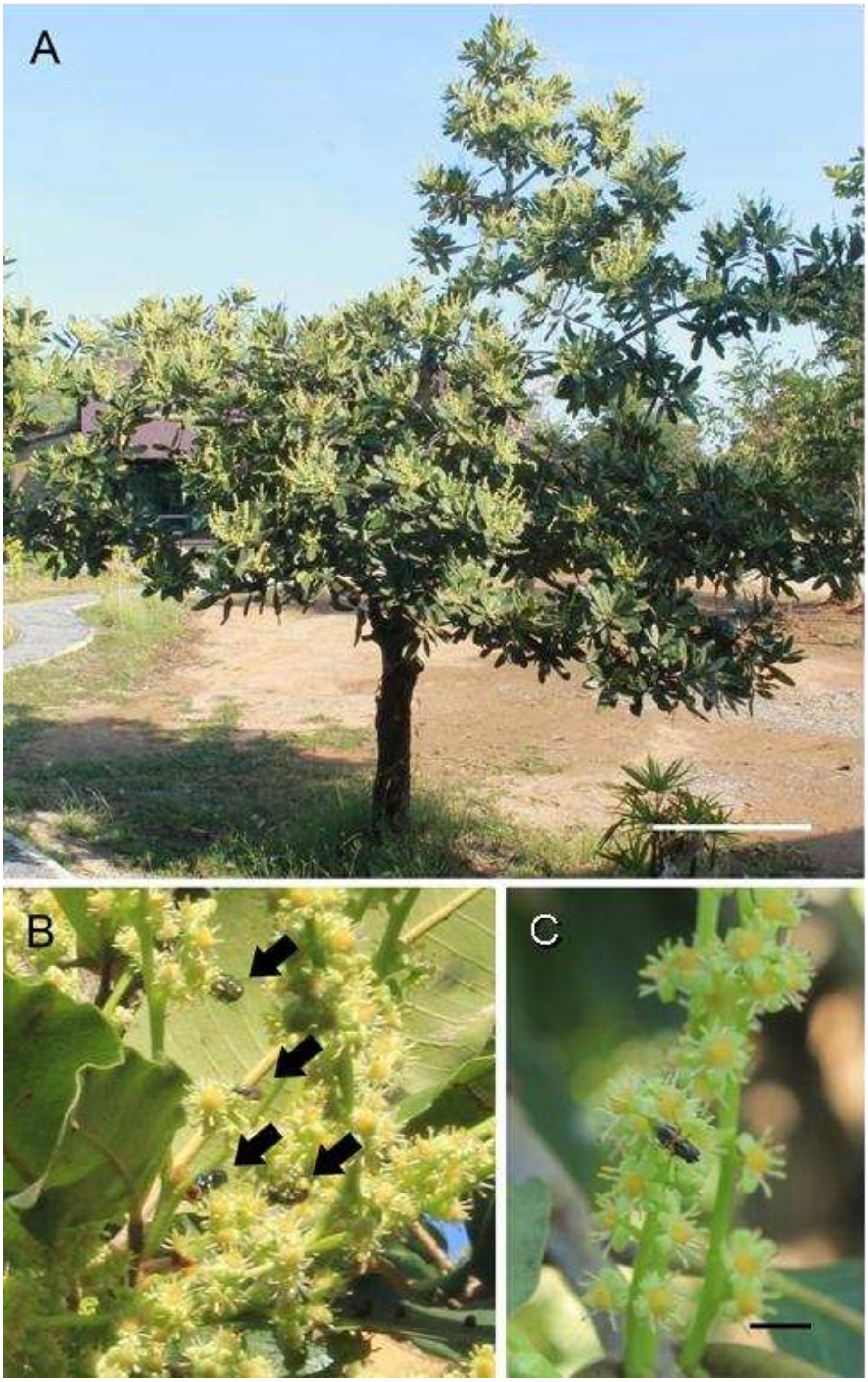

Figure 1. *The sampled Buchanania lanzan tree, located near the administrative offices of Mahidol University Nakhonsawan Campus, Nakhonsawan Province, Thailand; (A) the tree with flowers (bar = 1 m); (B) various blow fly species visiting the blossom (black arrow heads); and (C) Stomorhina discolor, the predominant species visiting blossoms (bar = 1 cm)*

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Data analyses

The numbers of individuals of each insect species collected from the *B. lanzan* tree were counted.

Statistical analysis was performed using SPSS 17.0 for Windows. ANOVA was used to determine the difference among numbers of flies (males vs. females) collected during morning (0900-1200 h) and afternoon (1300-1600 h) ($p < 0.05$).

Results

Diversity of insects on B. lanzan flowers

A total of 843 insects were collected during investigations (*Table 1*). The numbers of insects collected between years were quite similar, with 404 insects in 2013 and 439 insects in 2014. Insects were sorted into 3 orders. Flies (Diptera) ranked as by far the most abundant insects, while the combined number of bees (Hymenoptera: *Apis mellifera*) and beetles (Coleoptera) contributed only 3.7% of the total.

Table 1. *Classification and total number (%) of insect species visiting flower of Buchanania lanzan at Mahidol University Nakhonsawan Campus, during 2-day sampling of 27–28 December 2013 and 13–14 December 2014*

Order	Family	Species	No. (%)
Diptera	Calliphoridae	*Stomorhina discolor* (Fabricius, 1794)	199 (23.6)
		Chrysomya megacephala (Fabricius, 1794)	57 (6.8)
		Chrysomya rufifacies (Macquart, 1843)	30 (3.6)
		Ceynolomyia nigripes (Aubertin, 1923)	5 (0.6)
		Rhyncomya flavibasis (Senior-White, 1922)	2 (0.2)
	Syrphidae	Undetermined	156 (18.5)
	Stratiomyidae	*Hedriodiscus* sp.	116 (13.8)
	Drosophilidae	*Drosophila melanogaster* Meigen, 1830	96 (11.4)
	Muscidae	*Graphomya rufitibia* Stein, 1918	66 (7.8)
		Musca sorbens Wiedeman, 1830	19 (2.2)
	Phoridae	*Megaselia scalaris* Loew, 1866	12 (1.4)
	Sarcophagidae	*Boetcherisca peregrina* Robineuau-Desvoidy, 1930	4 (0.5)
		Parasarcophaga dux Thomson, 2867	3 (0.4)
		Sarcophaga spp. Meigen, 1826	2 (0.2)
	Tachinidae	Tachinid fly	7 (0.8)
	Tabanidae	*Tabanus* sp. Linnaeus, 1758	3 (0.4)
	Undetermined	Undetermined Diptera	35 (4.2)
Hymenoptera	Apidae	*Apis mellifera* Linnaeus, 1758	24 (2.8)
Coleoptera	Undetermined	Undetermined	7 (0.8)
Total			**843 (100)**

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Ten families of flies were found on *B. lanzan* flowers (*Table 1*). Of these, blow flies (Calliphoridae) were the most abundant (34.8% of total) and species-rich (R = 5 species). These comprised *Stomorhina discolor* (*Fig. 1B-C*), *Chrysomya megacephala* (*Fig. 1B*), *Chrysomya rufifacies, Chrysomya nigripes*, and *Rhyncomya flavibasis*.

Syrphid flies (Syrphidae) were the second most abundant (18.5% of total; R = 4) and comprised 4 morpho-species. *Hedriodiscus* sp. (Stratiomyidae), *Drosophila melanogaster* (Drosophilidae), and *Graphomya rufitibia* and *Musca sorbens* (Muscidae) all contributed high numbers in our samples. Otherwise, *Megaselia scalaris* (Phoridae), *Boetcherisca peregrina, Parasarcophaga dux, Sarcophaga* sp. (Sarcophagidae), and other undetermined Diptera were much less abundant. Blood-sucking flies (Tabanidae: *Tabanus* sp.) contributed just 3 individuals (*Table 1*).

Visitation of insects on B. lanzan flowers

The number of insects visiting the flowers of *B. lanzan* was higher during the morning (0900-1200 h) than the afternoon (1300-1500 h) (*Fig. 2*). The difference in the numbers visiting between morning and afternoon periods was significant in the blow flies *S. discolor* and *C. megacephala* (p = 0.002 and 0.023, respectively). Significant differences were also found in *Hedriodiscus* sp., *D. melanogaster*, *G. rufitibia*, and syrphid flies ($p < 0.05$). No significant difference was found in the diurnal visitation pattern of *A. mellifera* or beetles, while *R. flavibasis* and *Tabanus* sp. were only found during the morning period.

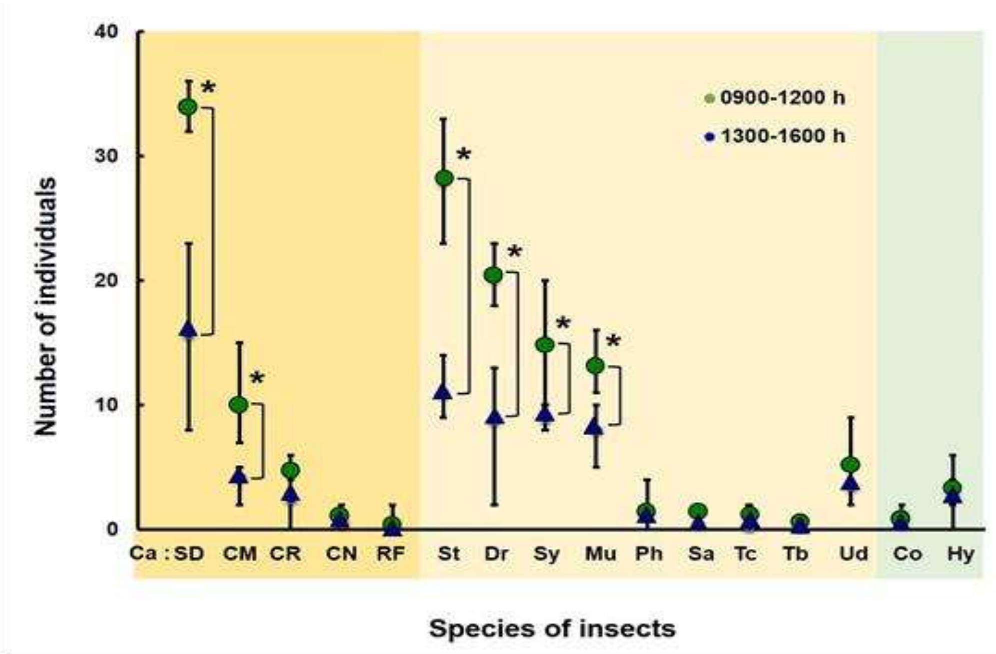

Figure 2. *Numbers and visitation times of insects sampled on flowers of Buchanania lanzan at Mahidol University Nakhonsawan Campus, during sampling, 27–28 December 2013 and 13–14 December 2014 (n = 4) (Ca Calliphoridae, SD Stomorhina discolor, CM Chrysomya megacephala, CR Chrysomya rufifacies, CN Chrysomya nigripes, RF Rhyncomya flavibasis, St Stratiomyidae, Dr Drosophilidae, Sy Syrphilidae, Mu Muscidae, Ph Phoridae, Sa Sarcophagidae, Tc Tachinidae, Tb Tabanidae, Ud undetermined Diptera, Co Coleooptera, Hy Hymenoptera); *indicates significant differences in numbers of insects visiting between 0900–1200 h and 1300–1600 h (independent T–test, p < 0.05)*

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More female flies were caught than males, although this difference was not significant ($p > 0.05$) in most. In muscid flies and sarcophagid flies males predominated over females ($p > 0.05$).

Occurrence of pollens on insect bodies

Forty-five percent of insects sampled had pollen on their bodies (*Table 2*). The exceptions were the tiny flies of *D. melanogaster*, *M. scalaris*, and *C. nigripes*, blood-sucking *Tabanus* sp., and beetles.

Table 2. *Percentage of insects with pollen on their bodies at Mahidol University Nakhonsawan Campus during periods of 27–28 December 2013 and 13–14 December 2014*

Order	Family or Species	% of insects with pollens	% of insects bear organs with pollens				
			Proboscis	Eyes	Thorax	Legs	Abdomen
Hymenoptera	*Apis mellifera*	75.0	0	0	50.0	37.5	33.3
Diptera	Tachinidae	71.4	14.3	0	57.1	57.1	42.9
	Graphomya rufitibia	59.1	10.6	13.6	34.8	30.3	18.2
	Stomorhina discolor	58.3	6.5	0	48.7	11.1	13.1
	Hedriodiscus sp.	54.3	0	0	31.9	24.1	15.5
	Syrphidae	52.6	3.2	5.1	30.8	23.1	26.3
	Rhyncomya flavibasis	50.0	50.0	0	50.0	0	0
	Chrysomya megacephala	43.9	14.0	0	12.3	33.3	24.6
	Musca sorbens	42.1	0	0	26.3	21.1	42.1
	Chrysomya rufifacies	40.0	23.3	0	13.3	6.7	26.7
	Sarcophagidae	33.3	0	0	11.1	33.3	11.1
	Undetermined Diptera	20.0	0	0	11.4	14.3	0
	Total	**45.0**	**5.0**	**2.0**	**28.8**	**18.0**	**16.5**

The small number of honey bees *A. mellifera* (24 bees) ranked first among the percentage of individuals bearing pollen (75%), found on thorax, legs and abdomen. They were followed by tachinid flies (71.4%) and *G. rufitibia* (59.1%), consecutively. *S. discolor*, the most abundant fly sampled in this study, ranked fourth (58.3% bore pollen). In flies of these three families pollen was often present on the proboscis in addition to thorax, legs and abdomens. Additionally, pollen grains were attached to the minute hairs located between the facets of compound eyes of both muscid flies, *G. rufitibia*, and syrphid flies.

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Discussion

The two years' observations revealed that *S. discolor* was the most common blow fly found around opened flowers of *B. lanzan*. The relationship between this fly species and the flowers of *B. lanzan* has not been previously documented. The length of the pollinator's proboscis is generally related to shape of the flowers they visit (Tangmitcharoen and Owens, 1997). A short proboscis, as found in flies and bees, is usually associated with feeding on already opened flowers (Larson et al., 2001). In addition, different insect pollinators are attracted to flowers of different colors and shades. Flowers with dull colors, such as those of *B. lanzan*, tend to attract fly pollination; while, bright colors mostly attract bees (Shivanna and Tandon, 2014).

In this study, flies preferred to visit flowers in the morning rather than in the afternoon, recalling the findings of Brown and McNeil (2009) for cloudberry *Pubus chamaemorus*. The peak of diurnal activity differs among different insect species and is usually correlated with the diurnal blooming peak of particular plant hosts. Peak blooming of mango, for example, tends to occur during 0900-1100 h (Huda et al., 2015). The timing of the blooming of *B. lanzan* in relation to *S. discolor* visitation requires further investigation.

Although pollen was found attached to the fly specimens collected in this study, whether any of these fly species is an actual pollinator of this tree is still in question. Banziger et al. (2008) found that despite the fact that blow flies (*Calliphora vomitoria* (Linnaeus) and *Calliphora pattoni* Aubertin) entered the lip of the lady slipper orchids *Cypridedium yunnanense* and *C. flavum* (Orchidaceae), they proved not to be pollinators, even when smeared with pollen. Nonetheless, there are other reports of blow flies as pollinators. For example, females of *Lucilia porphyrina*, *Chrysomya pinguis, Chrysomya chani* and *Chrysomya villeneuvei* were the main pollinators of *Rhizanthes zippelii* (Rafflesiaceae) in Thailand (Banziger, 1996). Howlett (2012) found that the European blue blow fly *Calliphora vicina* Robineau-Desvoidy was an effective pollinator of the hybrid carrot (*Daucus carota* L.) seed crop in New Zealand. In China, several species of blow flies (*C. megacephala, Isomyia isomyia, Pierretia* sp., *Hemipyrellia* sp. and *C. rufifacies*) were the most frequent visitors to flowers of *Bridelia stipularis* and *Cleistanthus sumatranus* on tropical Hainan Island (Li et al., 2014). Likewise, females of *Lucilia* and *Chrysomya* were major pollinators of *Rafflezia pricei* in northern Borneo of Malaysia (Beaman et al., 1988). Moreover, *C. megacephala* blow flies have been reared and used as pollinators of mango in Australia (Anderson et al., 1982) and Taiwan (Hu et al., 1995). In Hong Kong, China, Lau et al. (2009) revealed that *C. megacephala* provided more effective pollination of *Bauhinia* sp. (Leguminosae) than honey bees due to its more frequent rate of visitation. Greenhouse experiments would help verify whether *S. discolor* is truly a pollinator of *B. lanzan*.

In Thailand, the *B. lanzan* tree is little known although it is found in many parts, especially in the northeastern and central regions (Wongpakam et al., 2007; Sereesongsaeng and Khumchompoo, 2009). It is utilised by local people near Mahidol University Nakhonsawan Campus, who take the young leaves, young shoot tips and flowers of *B. lanzan,* eating them as a fresh vegetable with Thai dipping paste (chili paste).

Further, although the odor of *B. lanzan* flowers was not strong, unlike carrion flowers; it was remarkable that flies, including medically important blow flies such as *C. megacephala* and *C. rufifacies*, were attracted to visit. Therefore the odor-causing chemicals of *B. lanzan* flowers should be studied for possible application as a bait to trap those fly species when necessary for pest control. In conclusion, this study sheds

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light on the relationship of *B. lanzan* flowers and the blow fly *S. discolor* under natural conditions in Thailand. These new findings might be useful for further conservation management and economic production of *B. lanzan,* and also for the use of *B. lanzan* flowers such as the chemicals which they extrude as bait for controlling medically-important fly populations. Such information will help widen our knowledge of the roles of indigenous flies for improving agricultural practice in the future.

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THE GROUND BEETLE (COLEOPTERA: CARABIDAE) COMMUNITY IN AN INTENSIVELY MANAGED AGRICULTURAL LANDSCAPE

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Abstract. The effects of intensive agricultural management practices and environmental changes on biodiversity can be monitored by using the carabid beetles as biological indicators of agroecosystems quality. This study aimed to investigate the ground beetle species composition, abundance, dominance, diversity, zoogeographical types and distribution groups in an intensively managed agricultural field. Epigeic carabid fauna was collected weekly using pitfall traps on an arable crop field in Podravina, Croatia. Altogether, 1429 individuals belonging to 26 species and 15 genera were collected. The most abundant and eudominant were *Poecilus cupreus* (Linnaeus, 1758), followed by *Brachinus psophia* Audinet-Serville, 1821 and *Pterostichus melas melas* (Creutzer, 1799). Two species were dominant, two subdominant, four recedent and 15 subrecedent. The diversity of fauna was moderately high: Simpson diversity index 0.7875, Shannon-Wiener index 1.9654 and Pielou's evenness 0.6032. Zoogeographical analysis showed equal dominance of Euroasian and Palearctic species. Most (73%) of species belonged to E and 27% to A relict class. The majority of species were spring breeders (14 species), 8 species were autumn breeders and one species breeds in both seasons. In intensively managed agricultural landscape, ground beetle diversity was moderately high, because most of the species were eurytopic, i.e. capable of inhabiting strongly anthropogenically influenced landscapes.
Keywords: *Carabidae species composition, ecological factors, zoogeographical types, intensive crop production, agro-technical measures*

Introduction

Ground beetles are species rich and abundant in agricultural land all over the world (Lövei and Sunderland, 1996). As one of the most abundant and diverse groups overwintering within cultivated fields (Holland and Reynolds, 2003), they are often used in cultivation experiments. Carabids have also been successfully used for different kinds of indicator studies, serving as biological indicators of agroecosystems quality (Cole et al., 2002; O´Rourke et al., 2008). Most of these studies focus on beetles' response to agricultural management practices or changing environmental conditions (Rainio and Niemelä, 2003). According to Baranova et al. (2013), in terms of environmental quality, arable land represents an anthropogenically influenced, unstable and devastated biotope with low contribution to farmland diversity. Due to ground beetles' sensitive reaction to anthropogenic changes in habitat quality (Avgın and Luff, 2010), they have a bioindicative value for cultivation impacts, as well as for environmental change (Thiele, 1977; Maelfait, 1990).

Environmental change, through many abiotic and biotic factors, can cause different kinds of effects on the indicator species, including changes in species

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number and distribution (Blake et al., 1996; Rainio and Niemelä, 2003). Abiotic factors most often include temperature and soil moisture (Lövei and Sunderland, 1996; Holland, 2002). Other authors reported on many additional factors: landscape heterogeneity (Chapman, 2014), field size (Kromp, 1999), the presence of non-cropped habitat (Pollard, 1968; Sotherton, 1985) and land use diversity (Östman et al., 2001). Ground beetle abundance can be influenced by the crop-dependent timing of cultivation measures (Hence et al., 1990). According to Stassart and Grégoire-Wibo (1983), the depth of tillage is one of the major factors affecting the carabid fauna. Fertilization regimes (e.g. manure, mineral fertilizers) could also have a positive effect on ground beetle population (Pietraszko and De Clercq, 1982; Hence and Grégoire-Wibo, 1987) or a negative one (Kromp, 1990). Vician et al. (2015) considered the content of organic matter and pH as the most significant factors influencing ground beetle diversity in agroecosystems, while others stated soil particle size distribution can be a decisive factor in habitat selection (Thiele, 1977; Meissner, 1984).

Crop type can affect ground beetles through modification of microclimatic factors (i.e. temperature and humidity) and through disturbance factors (i.e. harvest and tillage schedules) (Thiele, 1977; Witmer et al., 2003; O´Rourke et al., 2008). The ground beetles population in the agricultural landscape can be also influenced by chemical pest control (Basedow, 1987; Asteraki et al., 1992; Jeschke et al., 2011; Szczepaniec et al., 2011; Varvara et al., 2012; Douglas et al., 2014).

Several studies in Croatia reported about epigeic ground beetles' assemblage, distribution and abundance in different vegetation types, including forests (Šerić Jelaska, 2005; Brigić et al., 2014a), wetlands (Brigić et al., 2014b), meadows (Durbešić, 1987; Durbešić et al., 2006) and parks (Durbešić, 1982; Marković, 2009). However, not many detailed studies about ground beetles on agricultural fields with intensive land cultivation have been done. Studies were performed on leguminous fields (Kovačević and Balarin, 1960; Balarin, 1974) and in wheat (Sekulić et al., 1973; Sekulić, 1977). The most comprehensive ground beetle faunal study on several different crop types was done in Podravina region more than 30 years ago (Štrbac, 1983), in which 31 species were identified. Since then, only few researchers investigated ground beetle assemblage in agricultural landscape, and these included research on sugar beet (Kos et al., 2013), maize (Kos et al., 2006; Bažok et al., 2007; Kos et al., 2011) and barley (Kos et al., 2010). The latest study on endogeic ground beetle communities in arable field in Podravina area revealed eight species (Drmić et al., 2016). Juran et al. (2013) investigated activity of the adult ground beetles in three differently managed fields in central Croatia and found that the endogaeic activity was highest in „organic" system, followed by the „conventional" and „integrated" system. Büchs et al. (2013) found 72 species on differently managed fields in a neighboring country. The authors, however, did not mention the species composition.

Different indices measure different aspects of the partition of abundance between species. Species evenness usually has been defined as the ratio of observed diversity to maximum diversity, the latter being said to occur when the species in a collection are equally abundant (Margalef, 1958; Patten, 1962; Pielou, 1966). Simpson's index, for example, is sensitive to the abundance only of the more plentiful species in a sample, and can therefore be regarded as a measure of "dominance concentration" (Whittaker, 1965). Used Shannon index is an

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information statistic index, which means it assumes all species are represented in a sample and that they are randomly sampled. This index estimates the affinity of different populations belonging to a community and, through the species composition, the similarity of the habitats (Popescu and Zamfirescu, 2004).

In modern intensively managed production in Croatia, there is still little knowledge on beneficial fauna importance (Bažok et al., 2015; Virić Gašparić et al., 2017). In order to preserve biodiversity in intensively managed arable land as much as possible, it is important to monitor the bioindicator species such as ground beetles, since they can indicate the anthropogenically influenced field quality. Detailed knowledge on their community in a specific agricultural landscape can give us a preview on agroecosystem stability. Therefore, this study aimed to investigate the ground beetle species composition, abundance, dominance and diversity, as well as zoogeographical types and distribution groups in an intensively managed agricultural field, with its specific agro-ecological factors and agro-technical measures.

Materials and methods

Location

Ground beetles were collected during the arable crop growing season in 2015 in Lukač (Virovitica–Podravina County, Croatia), on winter crop field with an intensive arable management and specific climatic and edaphic characteristics (field size 34.76 ha, coordinates: 45° 50′ 24″ N, 17° 24′ 0″ E). According to Köppen classification, this part of continental Croatia belongs to *Cfwbx* climatic type characterized with continental climate of cold winters and hot summers (Penzar and Penzar, 2000). The soils in the research area are gleyic luvisols (IUSS Working Group WRB 2015). These are hydromorphic soils, characterized by periodic or continuous wetting of part or whole of the profile, with stagnating precipitation or with additional surface or underground water that is not saline or alkaline. These soils contain a great amount of fine sand and coarse silt (sandy loams texture) (Bogunović et al., 1996) and often require conventional tillage.

The field was chosen to represent common cultivation practices as well as the agro-technical measures in this area. Considering the soil type and soil characteristics, the tillage was adapted to the given conditions and performed as follows: a) in autumn: ploughing on a depth of 20-25 cm was followed by the furrow closure for moisture conservation in spring; b) in spring: chisel ploughing and tillage with the rotary harrow; c) in summer: after harvest disk harrowing and again chisel ploughing. A description of the regional physical and chemical soil properties of investigated area as well as agrotechnical measures applied on the experimental field are given in *Table 1*. Performed pedological procedure consisted out of taking the soil sample from the depth of a plow layer (30 cm). Five sub-samples waging 300–400 g were taken and than pooled and homogenized for analysis. Analysis was performed by the pedology laboratory of the Department of Soil Science, Faculty of Agriculture, University of Zagreb and included sediment grain size and chemical properties analyses. Soil texture was determined by sieving following standard methods (ISO 11277 2004) (Kozina et al., 2015).

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***Table 1.** Physical and chemical soil properties of arable field where research was conducted*

Location	Soil type	Soil pH	Humus (%)	Soil properties (mm)	Fertilization	Bare soil (mth)*	Insecticide treatment
Lukač (Virovitica -Podravina County)	gleyic luvisol	KCl 5.58 H2O 6.65	3.2	Coarse sand 2.35 Fine sand 11.83 Coarse silt 38.42 Fine silt 31.65 Clay 15.75	74 kg N 60 kg P 90 kg K	2	Thiacloprid

*number of months while field was not covered after harvesting till soil preparing for crops grown in folowing vegetation season

Climatic factors

Climate data (i.e., mean weekly air temperature, mean weekly soil temperature and the total amount of rainfall per week) were obtained from the Croatian Meteorological and Hydrological Service and presented for ground beetle collecting period from May to September 2015 (19^{th} to 38^{th} week of the year).

Ground beetle trapping

Epigeic covered pitfall traps were used to collect adult ground beetles. Polythene pots (Ø=12 cm, h=18 cm) were incorporated 18 cm into the soil and covered with PVC roofs (Ø=16 cm) approximately 4 cm above ground level. Each trap was half filled with salted water (20% solution) for captures conservation. Four pitfall traps were placed into the center of the field at 50 m apart and 100 m away from the field edges. Trapping was performed from the 19^{th} to the 38^{th} week of the year, from May to September 2015. Traps were inspected once a week and all ground beetles were collected and counted. The identification of the collected ground beetles to species level was based on the work of Auber (1965), Bechyne (1974), Harde and Severa (1984) and Freude et al. (2006).

Ground beetle composition analysis

The ground beetle trapping results using pitfall traps for the selected interval (from 19^{th} to 38^{th} week of the year) are presented as a mean number of individuals caught per field per week. Results of the ground beetle population dynamics are presented as the total number of ground beetles caught per week as a function of the average weekly air temperatures (°C), total weekly precipitation (mm) and average weekly temperature of soil (°C) at a depth of 10 cm.

The dominance values of carabids presented in percentage shares of a particular species in the community were calculated according to Tischler (1949) as follows: eudominant (10-100%), dominant (5-10%), subdominant (2-5%), recedent (1-2%) and subrecedent (<1%). To calculate the diversity of the carabid assemblages, Simpson (λ) and Shannon-Wiener indices (H') were used. Shannon-Wiener indices is an entropy, giving the uncertainty in the outcome of a sampling process key (Jost, 2006). Both

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Shannon and Simpson diversities increase as richness increases, for a given pattern of evenness, and increase as evenness increases, for a given richness, but they do not always rank communities in the same order (Colwell, 2009). Evenness was estimated using Pielou's evenness. Analyses were carried out using the MATLAB program (The MathWorks Inc., 2015). Zoogeographical analysis adding new species records and contributing an understanding of the composition (Majka et al., 2007), was made according to Vigna Taglianti et al. (1999) and the database Fauna Europaea (Vigna Taglianti, 2013). The distribution/occurrence groups (relict classes E, A and R) were defined according to Hůrka et al. (1996).

Results and discussion

This study aimed at observation and description of a ground beetle fauna during one vegetation season in intensive arable crop production. During the sampling period, a total of 1429 individuals were collected using epigeic traps at Podravina region. Ground beetles collected belong to 26 species and 15 genera (*Table 2*) which in comparison with previous studies in arable agroecosystems can be classified as moderately high (Kos et al., 2006; Bažok et al., 2007; Kos et al., 2010, 2011; Drmić et al., 2016; Virić Gašparć et al., 2017). Despite the large number of species which may occur in agroecosystems, a relatively small number have been identified as being characteristic of arable areas and these are often the most abundant (Thiele, 1977; Holland and Luff, 2000).

The composition of recorded species in arable crops corresponds with results of similar investigations in Croatia (Kos et al., 2006; Bažok et al., 2007; Igrc Barčić et al., 2008; Kos et al., 2010; 2011; Drmić et al., 2016) and abroad (Bukejs and Balalaikins, 2008; Woodcock et al., 2010; Baranová et al., 2013). The most abundant species in the total catch was *Poecilus (Poecilus) cupreus cupreus* (Linneaus, 1758) (37.65%) followed by *Brachinus (Brachinus) psophia* Audinet-Serville, 1821 (21.06%) and *Pterostichus (Feronidius) melas melas* (Creutzer, 1799) (10.29%) (*Table 2*). The most abundant species accounted almost 70% of the total catch and belonged to the group of eudominant species. *Anchomenus (Anchomenus) dorsalis* (Pontoppidan, 1763) and *Harpalus (Pseudoophonus) rufipes* (DeGeer, 1774) were classified as dominant, *Amara (Amara) similata* (Gyllenhal, 1810) and *Pterostichus (Morphosoma) melanarius melanarius* (Illiger, 1798) as subdominant while others were recedent (4 species) or subrecedent (15 species). The species, which dominated the carabid assemblage in arable habitat (with the total collections), were *P. cupreus* (538), *B. psophia* (301), *P. melas* (147), *H. rufipes* (128) and *A. dorsalis* (97) (*Table 2*).

Species *P. cupreus* is considered as one of the most common species inhabitating winter crops (Alford, 2008), so these results strongly support this research. In Croatia, Štrbac (1983) also specified it among the three most dominant on arable land.

Drmić et al. (2016) investigated endogaeic ground beetle fauna in the same area in Croatia and detected *B. psophia* and *A. dorsalis* as the most abundant ones, therefore we may assume that these species are a typical arable ground beetle representatives in investigated region.

Species *P. melas* is also common in Croatia and was detected as dominant in agricultural land near the Nature park Lonjsko polje (Brigić et al., 2003).

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Table 2. *The composition, abundance, zoogeographical and geographical analysis of ground beetles collected in Lukač, 2015*

Species name	N†	DV‡	Zoogeographical categories and faunal types§	Geographical distribution groups∣	Reproduction period¶
Calosoma (Campalita) auropunctatum auropunctatum Herbst, 1784	1	0.07	E-CAS	A	no data found
Brachinus (Brachinus) crepitans Linné, 1758	27	1.89	B-CAS	E	Sp
Brachinus (Brachinus) psophia Audinet-Serville 1821	301	21.06	E-CAS	E	no data found
Brachinus (Brachynidius) explodens Duftschmid 1812	3	0.21	E-CA-M	E	Sp
Clivina fossor fossor Linné, 1758	13	0.91	E-AS	E	Sp
Asaphidion curtum curtum Heyden 1870	3	0.21	OLA	E	Sp
Trechus (Trechus) quadristriatus Schrank, 1781	4	0.28	E-CA-M	E	A
Anisodactylus (Pseudanisodactylus) signatus Panzer 1796	1	0.07	E-AS	E	Sp
Harpalus (Harpalus) affinis Schrank, 1781	1	0.07	E-AS	E	Sp
Harpalus (Harpalus) dimidiatus P. Rossi, 1790	1	0.07	E-PAS	A	A
Harpalus (Harpalus) distinguendus distinguendus Duftschmid, 1812	2	0.14	PAL	E	Sp
Harpalus (Pseudoophonus) rufipes DeGeer, 1774	128	8.96	PAL	E	A
Stenolophus (Stenolophus) teutonus Schrank, 1781	16	1.12	E-MED	E	Sp
Agonum (Amara) viridicupreum viridicupreum Goeze, 1777	1	0.07	E-PA-M	E	Sp
Anchomenus (Anchomenus) dorsalis Pontoppidan, 1763	97	6.79	PAL	E	Sp
Abax (Abacopercus) carinatus carinatus Dejean, 1828	4	0.28	E-PAS	A	no data found
Abax (Abax) parallelepipedus parallelepipedus Piller & Mitterpacher, 1783	1	0.07	EUR	A	A
Poecilus (Poecilus) cupreus cupreus Linné, 1758	538	37.65	E-AS	E	Sp
Pterostichus (Feronidius) melas melas Creutzer, 1799	147	10.29	E-PAS	A	A
Pterostichus (Morphosoma) melanarius melanarius Illiger, 1798	54	3.78	E-SI	A	A
Pterostichus (Platysma) niger niger Schaller, 1783	1	0.07	E-AS	A	A
Calathus (Calathus) fuscipes fuscipes Goeze, 1777	19	1.33	PAL	E	A/Sp
Calathus (Neocalathus) ambiguus ambiguus Paykull, 1790	1	0.07	E-AS	E	A
Amara (Amara) aenea Degeer, 1774	3	0.21	OLA	E	Sp
Amara (Amara) ovata Fabricius, 1792	28	1.96	PAL	E	Sp
Amara (Amara) similata Gyllenhal, 1810	34	2.38	E-AS	E	Sp

† N-number of individuals; ‡ DV-dominance index; § I. Northern Holarctic and Euro-Siberian faunal type: OLA - Holarctic; PAL - Palearctic; E-SI - Eurosiberian; II. European faunal type: EUR - European; E-PAS - European-Neareastern; III. Euroasiatic faunal type: E-AS - Euroasiatic steppe complex; E-CAS - European and Central Asian; B-CAS - Balkan and Central Asian; IV. Mediterranean (s. lato) faunal type: E-CA-M - European-Centralasian-Mediterranean; E-PA-M - European-Neareastern-Mediterranean; E-MED - Eastmediterranean (Vigna Taglianti et al. 1999, the database Fauna Europaea (Vigna Taglianti, 2013)); ∣ Relict classes: E-eurytopic species, A-adoptable species; ¶ A-autumn, Sp-spring.

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Kromp (1999) listed species *H. rufipes*, followed by *P. cupreus* and *P. melanarius* as the most abundant from agricultural fields of Eastern European countries, which is generally in accordance with our results. Similar investigations from Croatia (Bažok et al., 2007; Igrc Barčić et al., 2008; Kos et al., 2011) also stated species *H. rufipes* and *P. melanarius* in the group of the most abundant species in corn fields. Although they were not the most abundant species in our results, they were among species which generally dominated with the total scores.

This typically structured ground beetle community of arable land consists of a small number of dominant species represented with a large number of individuals and a large number of less commonly occurring species (subdominant, recedent and subrecedent) represented with a low number of specimens (Baranová et al., 2013).

The diversity of fauna was moderately high: Simpson (1-λ') diversity index was 0.7875, Shannon-Wiener index (H') was 1.9654 and Pielou's evenness was 0.6032. Analysis of faunal types (zoogeographical analyses) showed the dominance of Euroasian (23.08%) and Palearctic (23.08%) species which corresponds with climatic and geographic characteristics of the investigated area (*Table 2*).

With reference to relict classes, 73% of determined ground beetles belonged to E relict class which consists of eurytopic species without special demands on habitat type and quality, and inhabiting strongly anthropogenically influenced landscapes (Hůrka et al., 1996). Species which belonged to A relict class were represented with 27% and this group included more adoptable species, which are found in more or less natural habitats (forests, meadows, pastures, standing and flowing water) (Hůrka et al., 1996). Neither one species was classified to relict class R, which was expected, because R class includes species with narrow ecological amplitude, which are rare and endangered, occurring naturally in undisturbed ecosystems which was not the case in our study (Hůrka et al., 1996). These results correspond to the results of Porhajašová et al. (2004) and Baranová et al. (2013) who reported that increasing human disturbances changes the composition to favor eurytopic species while reducing the number of specialized species with narrow ecological valences.

Abundance and diversity as well as the ratio of spring to autumn breeders varied between winter sown crops (cereals and oilseed rape) and spring sown root crops (potatoes, sugar beet, maize, carrots) (Kabacik-Wasylik, 1975 cit. Holland and Luff, 2000). Winter crops usually have higher abundance, diversity and more spring breeders with summer larvae (e.g. *P. cupreus, A. dorsalis*) which was confirmed with our results as well. These preferences are not, however, always apparent and even total numbers may vary (Holland and Luff, 2000). The majority of collected species were spring breeders (14 species), 8 species were autumn breeders and one species (*Calathus fuscipes fuscipes* (Goeze, 1777)) breeds in both seasons (*Table 2*). The domination of spring breeders could be a consequence of the cultivation measures. The depth of tillage is one of the major factors affecting field carabid communities, with superficial ploughing enabling a higher number of species and favoring spring breeders (Kromp, 1999 cit. Stassart and Grégoire-Wibo, 1983).

Species composition and the number of ground beetles in different agrocenosis differ and depend on edaphic factors (Bukejs and Balalaikins, 2008). Ground beetle species contribute significantly to the insect diversity in farmland because many species are adapted to agriculture and generally occur at high densities (Booij, 1994). According to Thiele (1977) and Kromp (1999) cultivated land is comprised of widely distributed, eurytopic ground beetle species, many of which have high tolerance to disturbances and

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chemical pollution. This means that cultivated land contains a typical ground beetle fauna, despite the regular implementation of cultivation measures (Kromp, 1999). For example, Thiele (1977) listed 26 species found at investigated arable habitats stretching from England over Central Europe.

In our survey the first population maximum was observed from week 19th to 21st which was also the beginning of sample collection period. The second population maximum was recorded from week 32nd to 36th (*Figure 1*). Presented results of ground beetle population dynamics show that population increase follows air and soil temperature decrease (*Figure 1*). In the whole investigation period the number of ground beetle decrease is followed by precipitation increase. Recording to Croatian Meteorological and Hydrological Service the Virovitica-Podravina County is described as mid worm area with intensive periods of rainfall especially in summer period.

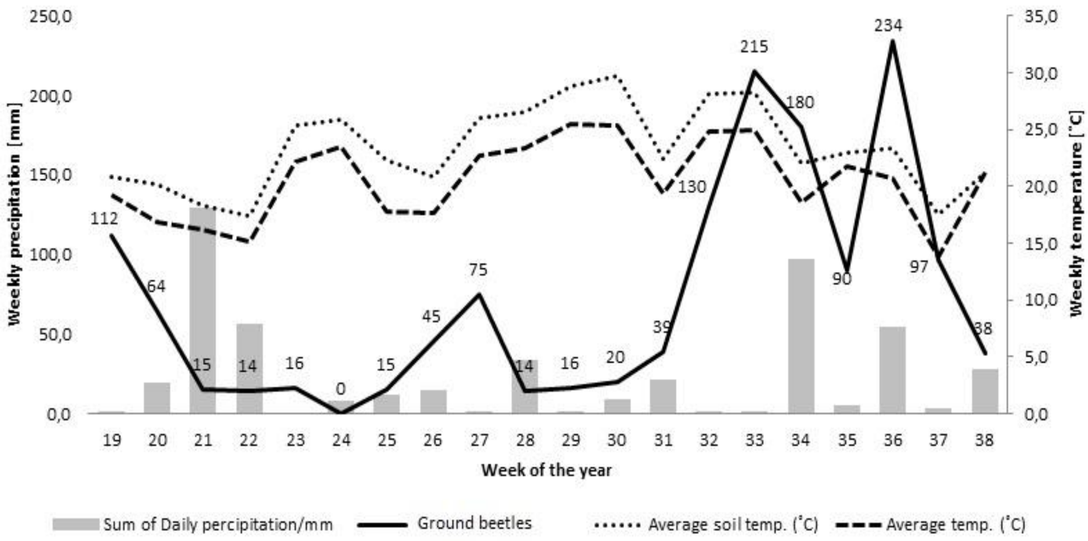

Figure 1. *Ground beetles weekly dynamics with prevailing climatic conditions*

According to edaphic factors prevailing at investigation area ground beetles inhabited slightly acidic soil with a great amount of fine silt and a small proportion of clay (*Table 1*). The intensity of ploughing was the main agro-technical specificity at studied locality. The field in Podravina has been ploughed often and on great depth during whole vegetation season. Ploughing is known to significantly influence physico-chemical and biological soil properties and affects the abundance of invertebrates (Vician et al., 2015). Generally, reduced soil disturbance, increased surface residues and greater weed diversity had positive impact on invertebrates (Kromp, 1999). According to previous studies, higher ground beetle trapping rates were recorded on fields with reduced tillage or no tillage at all compared with conventionally tilled ones (House and All, 1981; Blumberg and Crossley, 1983; House and Stinner, 1983; Ferguson and McPherson, 1985; House and Parmalee, 1985; Stinner et al., 1988; Tonhasca, 1993). Conventional tillage, such as conducted on the field in Podravina, could have an impact on established ground beetles abundance.

The soil factors are greatly influenced by weather conditions and ploughing but also could be affected by crops growing at the area. Previous studies have shown that microclimatic factors, such as temperature and humidity, and disturbance factors such

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as harvest and tillage schedules crops affect ground beetles communities (Thiele, 1977; Holland, 2002). Although no ground beetle species appears to be strictly bound to a certain crop, early agro-ecological studies in Europe reported a general difference between ground beetle abundance distributions in winter versus spring crops (Heydemann, 1955). O´Rourke et al. (2008) stated that thick stand winter crops provide important refuges for ground beetles in comparison with spring crops. The overwintering crop sown at the field in our survey may confirm the importance of crop habitat for supporting ground beetle populations by providing less extreme microhabitat in spring and creates positive conditions for ground beetle survival and the dominance of spring breeders.

Beside crop specifics, bare soil period can also be a significant factor that affects ground beetle communities. In winter crops, the less extreme microclimate already established in early spring creates favorable conditions for ground beetles (Kromp, 1999). Locality in our study had a very short period of bare soil (2 months period without plant cover; *Table 1*). No negative effect was observed in ground beetle populations regarding the extreme soil surface microclimate. The effect of intensively managed crop on ground beetles abundance which could be detected in this research support the results of numerous other studies (Tonhasca, 1993; Zhang et al., 1998; Honek and Jarosik, 2000; Ward and Ward, 2001; Witmer et al., 2003; O´Rourke et al., 2008).

As well, the fertilization in Podravina is generally intensive while insecticide treatments were common and in compliance with IPM. While previous studies had concluded that insecticides have negative influence on the ground beetle populations (Asteraki et al., 1992, 1995), more detailed investigation are needed for the full conclusion. Kromp (1999) shown that high amount of nitrogen used in fertilization process decrease ground beetle abundance. The levels of nitrogen applied in Podravina are under permitted levels (EU Directive 2009/128/EC, EUR-Lex, 2009) causing minimal negative influence on ground beetles. Only mineral fertilization has been used in Podravina so possible positive effect of organic manure recorded by Pietraszko and De Clercq (1982) and Hence and Grégoire-Wibo (1987) on ground beetle communities cannot be discussed.

Conclusions

The bioindicator species such as ground beetles have not received much attention by researchers in Croatia, although they can indicate the anthropogenically influenced field quality. In this study we gained detailed knowledge on their community in a specific agricultural landscape in northwest Croatia, Podravina region. In this investigation, a total of 1429 ground beetles were collected using epigeic traps, belonging to 26 species and 15 genera. Ground beetle diversity was moderately high, because most of the species were eurytopic, i.e. capable of inhabiting strongly anthropogenically influenced landscapes. In modern agriculture in European Union, conservation programs aimed to keep beneficial species and biodiversity are promoted as tool for ensuring sustainability. In order to measure the success of such programs, one has to have detailed knowledge on the initial situation. The results of this study significantly contributed to better understanding of initial situation about ground beetle communities in intensive agricultural landscape in northwest Croatia and will be a good entry point for future conservation programs.

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DETERMINATION OF CARBON EMISSIONS IN SHALLOW SOIL OF HARRAN PLAIN, TURKEY

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Abstract. Global climate change is known to be a result of atmospheric greenhouse gases. Thus, the atmospheric greenhouse gases should be reduced to prevent the climate change. Based on this fact, scientists have been working to reduce atmospheric greenhouse gases (CO_2, N_2O, CH_4). Due to the increases in CO_2 emissions in agricultural lands, the interest in scientific research had moved to this field. In the present study, the shallow soil CO_2-C emissions in Harran Plain were measured. The soil CO_2-C emissions were measured weekly using the soda-lime method. The Mean soil CO_2-C emission was 8.70 ± 0.57 g m^{-2} $week^{-1}$, according to the results obtained in the study. Climate data from the same period obtained that soil moisture was 17.21 ± 2.05% and soil temperature was 12.87 ± 1.80°C. A very significant positive correlation was found between soil CO_2-C emissions and soil temperature ($r = 0.049$; $p < 0.01$) and there was very significant negative correlation between soil CO_2-C emissions and soil moisture ($r = -0.196$; $p < 0.01$). There was a very strong statistically significant negative correlation ($r = -0.854$, $p < 0.01$) between soil moisture and soil temperature. While the soil CO_2-C emissions decreased as the soil moisture increased, it increased with the soil temperature.
Keywords: *atmospheric greenhouse, carbondioxide emission, terrestrial ecosystems, agricultural activities*

Introduction

Soil respiration, which is an important part of the respiration in the ecosystem, is one of the most important processes in atmospheric carbon transport from terrestrial ecosystems to the atmosphere. (Fiedler et al., 2015). Carbon equilibrium in these ecosystems was defined as the balance between plant carbon loss due to plant and soil respiration and plant carbon intake (Beer et al., 2010; Luyssaert et al., 2007; Malhi et al., 1999; Le Quéré et al., 2009, 2014; Trumbore, 2006). The equilibrium value, in other words, carbon storage and carbon resource behavior of terrestrial ecosystems has been of great interest in climate change studies (Hashimoto et al., 2015).

Since the carbon cycle is a bio-geochemical phenomenon, it was stated that it is very difficult to predict the CO_2 exchange between the biosphere and the atmosphere due to the complex relationships between physical, chemical and biological processes (Friedlingstein et al., 2006). Despite this complexity, carbon cycle studies and CO_2 measurements are conducted with different methods at the present time. Patra et al. (2013) stated that verification of estimates could only be done at the point scale, in addition to the complexity of the system, because reliable data can only be obtained on site observations. Today, measurements are generally conducted with chamber-based

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methods. After measurements were taken, basic modeling was used and the CO_2 emissions were estimated in the period between measurement dates (Beetz et al., 2013).

The main greenhouse gases that cause climate change and global warming are CO_2, CH_4 and N_2O (Wu and Lee, 2011). The fossil fuel use, deforestation, industrial growth and agricultural activities increase atmospheric greenhouse gas emissions. The most important of these gases that have increased in the atmosphere since the industrial revolution, is CO_2, which has increased by about 30% since 1750. It is estimated that the amount of CO_2 was 280 ppm before the industrial revolution and it was 370 ppm in 1999, and 380 ppm in 2007 (Houghton et al., 2007).

Efforts to determine the amount of soil CO_2 emissions due to agricultural activities are increasing rapidly. Sakin and Sakin (2015) determined the average CO_2 emissions in the clay soil of Sanliurfa-Harran Plain located in the arid and semi-arid Southeastern region in Turkey as 5.46 g m^{-2} day^{-1} at 0-5 cm depth based on soil temperature and moisture. A positive correlation between soil CO_2 emission and soil temperature, and a negative relationship between soil CO_2 emission and soil moisture were also found in the abovementioned study. Zhou et al. (2015) reported that an increase in soil temperature and microorganism activities caused increased decay and decomposition.

High emissions values were determined in studies conducted in arid and semi-arid environmental conditions. Allaire et al. (2012) recorded 9.31-11.5 g CO_2 m^{-2} day^{-1} emissions in semi-arid climate shrubland and shrubbery and 5.48 g CO_2 m^{-2} day^{-1} emissions in grasslands. Weekly CO_2 emissions fluctuated during the growth season in a study conducted in pasture areas in arid regions of China, and it was determined as average 2.4 g CO_2 m^{-2} day^{-1}. In semi-arid regions in Spain, 4.07-15.43 g CO_2 m^{-2} day^{-1} emission was recorded (Curiel Yuste et al., 2003)

Sakin (2016) measured the CO_2 emissions based on soil temperature and moisture in semi-arid uncultivated soil in the Southeastern region in Turkey. Based on that study, the soil moisture at 5 cm soil depth was 11.66% and the soil temperature was 22.53°C, while soil CO_2 emission was 7.94 g m^{-2} day^{-1}. Soil CO_2 emissions has a positive correlation with soil temperature and a negative correlation with soil moisture. In the study, the maximum CO_2 emissions were observed in February and the minimum emissions was observed in August. The soil CO_2 emissions demonstrated annual fluctuations based on climatic parameters. It was stated that the climate change has emerged as a result of annual fluctuations.

Celik et al.(2017) reported in arid and semi-arid climate regions, soil carbon content is generally low because of the factors such as insufficient precipitation, low biomass and organic carbon intake, high oxidation, use of soil biomass as animal feed, being picked up for burning or as fuel. On the other hand, clayey nature of the soils is significant in terms of SOC content and accumulation. Clay surrounds SOC, and creates organo-mineral complexes, and protects the soil against decomposition and disintegration.

Turkey has quite large agricultural areas. The objective of the present study was to determine shallow soil CO_2 emissions due to climatic factors in Southeastern Turkey.

Material and Method

Study area

The study area is a part of “Harran Plain” on South East of Turkey. It is located between 37 ° 10 '14' 'N latitude and 39 ° 00' 14 " E longitude (*Fig. 1*). The altitude of the test area, which is located on a flat and near-flat topography, is 507 m. The region's

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summers are arid and hot, and winters are temperate and with low-precipitation. Precipitation is not sufficient for agricultural cultivation during certain years. The mean annual precipitation is 448.11 mm, the annual average maximum temperature is measured as 41.12°C in July, and the lowest temperature is measured as 2.41°C in February. Annual mean relative humidity was the highest at 92.32% and the lowest at 33.29% (TSMS, 2016).

Figure 1. *Location of the study area*

Laboratory analyses

It was determined that the soil reaction (pH) of the experimental soil was 7.30, electrical conductivity (EC) was 52.60 μS cm^{-1}, organic carbon content was 0.8%, cation exchange capacity was 35.15 cmol kg^{-1}. Among the exchangeable cations, calcium amount was the highest and potassium amount was the lowest. The particle fractions of the soil included mostly clay-sized particles.

The amount of soda-lime considered in the studies varies based on the ecological conditions, but 50 g of soda-lime was taken for the present study. The study was set up in the Faculty of Agriculture experiment area in 3 replicates and the calculations were made based on the following equation.

$$E_{CO_2} = \frac{(SL_{ad} - SL_{ini}) \cdot WC}{(A \cdot t)} \quad \text{(Eq.1)}$$

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Where;

E_{CO_2} is the total amount emitted during the incubation period (g CO_2 m^{-2} day^{-1});

SL_{ad} is CO_2 adsorbed soda-lime amount (g);

SL_{ini} is the initial soda-lime amount (g);

A is the area of measurement (m^2);

t is the incubation time (the time past in the field) (day) and;

WC is water correction number was taken as 1.69.

Results and Discussion

Weekly soil CO_2-C emissions varied between 6.21-17.22 ± 0.57 g m^{-2} $week^{-1}$, soil temperatures varied between 5.33-28.28±1.80°C and weekly soil moisture varied between 7.05-29.89 ± 2.05 (*Table 1*). As the soil moisture increased, soil temperature decreased, and as a result, soil CO_2 emissions decreased. Maximum soil CO_2-C emission was determined when the soil moisture was 7.71% and soil temperature was 18.58°C, while the minimum emission was determined when the soil moisture was 29.89% and soil temperature was 6.39°C. Soil emissions are not only dependent on soil temperature and moisture factors but also on other soil properties. Soil CO_2-C production was reported to be the result of plant root respiration and microbial degradation of organic carbon, and these factors were dependent on the soil temperature and moisture (Davidson et al., 2006). In previous studies conducted in the region, it was reported that the soil emissions varied based on the soil temperature and moisture, consistent with the findings of the present study (Sakin, 2016; Sakin et al., 20015, Sakin and Sakin, 2015; Gülle Sakin et al., 2015).

Soil is a heterogeneous medium that consists of gas, liquid and solid phases and varies in depth. The CO_2 transport in the soil occurs straight from the bottom to the soil surface. Thus, the CO_2 transported to the surface is released to the atmosphere. Soil CO_2 density varies based on the depth of the soil. In general, deep soil has a high CO_2 concentration, while in exposed (shallow) soil it is low. The soil CO_2 density in deep soil is about 100 times (6% -8%) the density on the soil surface. However, the amount of CO_2 resulting from the activities of organisms and roots is higher in the surface layer than in the sub layer (Allaire et al., 2012). Since the surface of the soil is rich in organic waste, life prospers more in this layer. Comparisons that were conducted in the present study confirmed this fact. In uncultivated fields, soil CO_2 emissions were about 20% higher than the cultivated areas in the current study.

Table 1. *Descriptive Statistics of soil factors (weekly.*

	Minimum	Maximum	Mean	Std. Error	Std. Deviation
CO_2 emission (g m^{-2} $week^{-1}$)	6.21	17.22	8.70	0.57	2.40
Soil Moisture (%)	7.05	29.89	17.21	2.05	8.68
Soil Temperature (°C)	5.33	28.28	12.87	1.80	7.64

In a study conducted in clay textured vineyards in Harran Plain, weekly soil CO_2-C emissions based on soil moisture and temperature (5.81-21.23 and 5.65-40.88°C) were measured between 4.94 - 20.97 g m^{-2} $week^{-1}$. It was stated that the effect of soil moisture and temperature on soil CO_2-C emissions cannot be ruled out, but the emission

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is not entirely dependent on these two parameters (Carlisle et al., 2006; Gaertig et al., 2002; Davidson and Janssens , 2006; Ricardson et al., 2012). CO_2 emissions were measured in an incubation study that was conducted to determine the correlation between CO_2 emissions in arid and semi-arid sites and the temperature and humidity factors influencing these emissions. Soil specimens were incubated under different humidity and temperature conditions and it was found that soil CO_2 emissions increased by 50% due to different moisture regimes where the soil temperature was kept constant (Conant et al., 2004).

The correlation between the weekly soil CO_2-C emissions, soil moisture and soil temperature is given in *Tables 2* and *3*. Based on this information, there was a strong negative correlation between the soil carbon emission and soil moisture ($r = -0.196$; $p < 0.01$) and there was a positive correlation between the soil carbon emission and soil temperature ($r = 0.049$; $p < 0.01$). As the soil moisture increased, soil temperature and activity decreased, and the opposite was true for soil temperature. Soil temperature and moisture should be at a suitable level for living organisms in the soil. It is considered that living conditions of plant roots and organisms are suppressed at extreme conditions.

Table 2. *Correlations between soil factors*

Soil factors		Soil Moist. (%)	Soil Temperature (°C)
CO_2 emission (g m^{-2} week^{-1})	Pearson Correlation	-0.196	0.049
	Sig. (2-tailed)	0.00	0.00
Soil Moisture (%VWC)	Pearson Correlation	1	-0.854
	Sig. (2-tailed)	0.00	0.000

Table 3. *One-Sample Test between factors*

Soil factors	Test Value = 0					
					95% Confidence Interval of the Difference	
	t	df	Sig. (2-tailed)	Mean Difference	Lower	Upper
CO_2 emission (g m^{-2} week^{-1})	15.38	106	.000	8.70	7.51	9.89
Soil Moisture (% VWC)	8.41	106	.000	17.21	12.89	21.53
Soil Temperature (°C)	7.14	106	.000	12.87	9.07	16.67

One of the important sources of soil respiration is roots and the second is the microbial respiration (Hanson et al., 2000) and 50% of soil respiration is attributed to plant root respiration (Hanson et al., 1993). Carbon decomposition and decay and CO_2 emissions by organisms are dependent on temperature and it was suggested to determine this correlation (Davidson et al., 2006). It was stated that the optimum temperature for soil respiration and organism activities, 35°C (Cable et al., 2011) is sufficient, however in certain areas the temperature is over 35°C. There are also researchers who argued that the optimum temperature for optimum soil respiration should be above 35°C (Barraon-Gafford et al., 2011).

In the arid and semi - arid Southeastern Turkey, the soil carbon (C) emissions based on soil moisture (12.89 ± 0.56) and soil temperature (18.19 ± 2.07) was measured in the clayey soil of Sanliurfa - Harran Plain. There was a positive correlation between the soil

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CO_2-C emissions and the soil temperature, and a negative relationship between soil CO_2-C emissions and the soil moisture. An increase in soil temperature resulted in an increase in microorganism activities, decomposition and decay (Zhou et al., 2015). Nobrega et al. (2016) reported that high soil carbon emissions were not related to the high carbon content in the soil, but rather were influenced by anthropogenic factors, however these factors prevented carbon intake of the soil. As a result, the soil carbon emissions results from carbon decomposition by the plant roots and microorganisms in the soil. Buerkert et al. (2012) found that soil carbon emissions were 3-5 times the initial amount after the first irrigation.

Sakin et al. ($P < 0.05$) determined a significant positive correlation between CO_2 emissions and relative moisture and the moisture inside the PVC container ($p < 0.05$), and a very significant negative correlation between CO_2 emissions and relative temperature and PVC container internal temperature ($p < 0.01$). An inverse correlation was identified between the relative humidity, relative temperature and PVC container internal temperature. In a study by Akburak (2008) conducted in the Belgrade forest in Istanbul, a weak positive correlation was found between soil CO_2 emissions and soil moisture and a negative relationship between soil CO_2 emissions and soil temperature. When soil respiration was above 10% of soil moisture content, a positive correlation was found between soil CO_2 emissions and soil temperature ($p < 0.01$). On the other hand, there was a positive correlation between soil water content and respiration when soil temperature was above 16°C in all land uses ($p < 0.001$). In dry periods, there was a negative relationship between soil emissions and soil temperature (Almagro, 2009). Similar results were obtained in the current study.

Conclusion

A very significant negative correlation was found between soil CO_2-C emissions and soil moisture and a very significant positive correlation was found between soil CO_2-C emissions and soil temperature. There was a very strong statistically negative correlation between soil moisture and soil temperature. The soil CO_2-C emissions decreased as the soil moisture increased while it increased as the soil temperature increased.

At the point where the soil moisture and temperature are at their maximum, soil CO_2 –C emissions are neither maximum nor the minimum. The soil CO_2-C emissions are the result of microbial activities of the organic carbon and plant roots in the soil, and the activities of the organisms that live in the soil. Thus, soil temperature and soil moisture should be optimum for the living beings in the soil.

The soil CO_2-C emissions increase or decrease due to the suppression of the optimum conditions for the living organisms in the soil when the soil temperature and the soil moisture are minimum and maximum. In studies similar to the present one, the objective is to establish measures to determine and then reduce CO_2-C emissions. If the soil CO_2-C emissions decrease with the increase in soil moisture, it does not mean that we should keep the soil submerged in water. Or, since the emissions are at the minimum under the maximum soil temperature, we cannot keep the soil constantly hot and without moisture. Thus, the idea of reducing the soil CO_2-C emissions when the optimum conditions for the living beings in the soil are not available should be developed.

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Appropriate agricultural techniques should be used to reduce soil CO_2-C emissions such as no - tillage, direct sowing and conservation agriculture techniques. Carbon dioxide measurements from soil should be made more healthful with modern equipment and more precise work. In arid and semi-arid climate regions, atmospheric CO_2-C should be given to the context of atmospheric CO_2-C to the soil through irrigation.

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HYDROLOGICAL IMPLICATIONS OF CLIMATE CHANGE ON RIVER BASIN WATER CYCLE: CASE STUDIES OF THE YANGTZE RIVER AND YELLOW RIVER BASINS, CHINA

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Abstract. As the two largest rivers in China, the Yangtze and Yellow Rivers are of great importance for water cycle and hydrological processes over the country, which have been significantly affected by climate change. In this study, by assessing the suitability of multiple GCMs (General Circulation Models) recommended by IPCC (Intergovernmental Panel on Climate Change), SDSM (Statistical Downscaling Model) and ASD (Automated Statistical Downscaling) were used to generate future climate change scenarios, which were used to drive VIC (Variable Infiltration Capacity) and SWAT (Soil and Water Assessment Tool) models to quantify climate change impacts in the Yangtze River Delta region and upper reaches of the Yellow River basin, respectively. Results showed that suitability assessment method adopted in this study coupled with statistical downscaling could effectively reduce uncertainties of GCMs. Compared with the baseline period (1961-1990), projected annual runoff in future periods (2046-2065 and 2081-2100) would decreased by 9.5% and 3% in the Yangtze River Delta region. While annual runoff in the upper Yellow River basin during future two periods would decrease by 16.9% and 22.2%. The results are of great significance to future water resources management and sustainable development under climate change in major river basins of China.
Keywords: *statistical downscaling, distributed hydrological model, changing environment, scenario analysis, Tibetan Plateau*

Introduction

The Fifth Assessment Report (AR5) of the Intergovernmental Panel on Climate Change (IPCC) pointed out that climate change has become an indisputable fact that will greatly threaten global and local water resource security (Stocker et al., 2013). In China, the tendency of climate change has been generally consistent with its global pattern (Qin, 2003; Qin et al., 2007).The multiscale, all-dimension, multi-level influences of climate change on humans, the ecology and environment (Hadson and Jones, 2002; Varis et al., 2004; Burn et al., 2010; Elfert and Bormann, 2010; Karamouz et al., 2011; Jung and Chang, 2011; Marvel and Bonfils, 2013; Jeong et al., 2014; Xu et al., 2015) are key risk factors for global sustainable development. Because the water cycle connects humans, the ecology and environment, its response to future climate change has become a focus of scientists, the general public, and decision-makers all over the world.

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China has a vast territory and various climate types. Because of substantial differences in geological environment, climate, and economic development stages in various regions, the influences of climate change vary. In the context of global warming, the frequency and intensity of climate extremes such as high temperature, low temperature, heavy precipitation and droughts in China had varying tendencies and regional differences (Piao et al., 2010). Recently, a number of researchers have conducted extensive studies of climate change and its effects on water resources in China. In general, it was found that over the past 100 years, hydrological climate elements changed substantially in major basins of the country, showing substantial temperature rises in most areas. In the last 50 years, there has been an uneven south-north precipitation distribution in the eastern monsoon region (Ye and Huang, 1996; Fu et al., 2005; Ding and Ren, 2008; Ding et al., 2006) and reduced basin runoff in the majority of northern exorheic rivers, aggravating water resource supply-demand conflicts in the north and pressure for flood control in the south (Chen, 2002; Liu, 2004; Qian et al., 2007). In most regions of the country, the potential (water surface) evaporation capability decreased dramatically (Qiu et al., 2003; Yang et al., 2003; Liu et al., 2004; Ren and Guo, 2006). Future climate change might exert strong impacts on water resources in China (Wang et al., 2002; Liu, 2007; Zhang and Liu, 2000; Xia et al., 2008), with reduced runoff in northern rivers but increased runoff in southern ones (Lin et al., 2006). Annual average evaporation may be on the rise (Wang et al., 2003), as may be the case also for the frequency of flood and drought disasters, which would further increase the vulnerability of water resources and conflict between water supply and demand (Lin et al., 2006).

Because of water cycle and water resources pattern changes in major river basins of China and prominent water issues such as flood disasters in the south and water shortages in the north, it is of great scientific importance and value to study the mechanism and spatiotemporal variation of the continental water cycle under the background of climate change. This will help assess the influence of such change on the basin water cycle and ensure sustainable socioeconomic development in the country.

In the current study, the Yangtze River Delta region and the upper reaches of Yellow River basin were selected to represent typical southern and northern basins in China. A multi-criteria score-based method was used to assess the suitability of multiple General Circulation Models (GCMs) provided by the IPCC. Screened GCM output results were downscaled to create future climate change scenarios suitable for the Yangtze and Yellow River basins. These scenarios were used to drive distributed hydrological models to simulate the spatiotemporal variation of water cycle elements in the study area, thereby evaluating the response of the river basin water cycle to future climate change.

Materials and Methods

Study area

The Yangtze is the longest river in Asia and the third longest in the world with a length of over 6300 km. Its drainage area, the Yangtze River basin, is located between 24°30′-35°45′N and 90°33′-122°25′E, spanning a total area of ~1.8×10^6 km^2. The Yangtze River basin stretches from the eastern Tibetan Plateau to the East China Sea and crosses 19 provinces in China (*Fig. 1*). The economy of the Yangtze River basin contributes 41.1% of China’s gross domestic product, and the area supports 34% of the nation’s population (Xu and Ma, 2009). Most of the YRB belongs to the East Asian

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monsoon region, which is sensitive and vulnerable to climate change (Fang et al., 2010; Xu and Ma, 2009). Human-induced land use/land cover change and climate change are now having enormous impacts on its water cycle (Zhang et al., 2014).

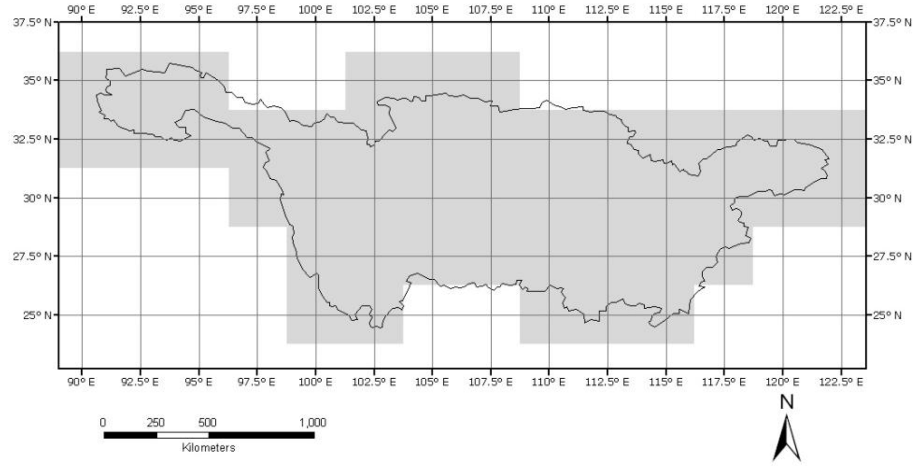

Figure 1. *Geographical boundary of Yangtze River basin with grids distribution used for GCMs*

The Yellow River is the second largest river in China with a drainage area of 7.95 × 105 km^2 (*Fig. 2*), and its topography is highest in the west and the lowest in the eastern parts of the Yellow River basin. The basin is located at mid-latitudes with a different climate prevailing in the southeastern part (higher precipitation) as compared to the northwestern part (lower precipitation). In addition, 91.93% of the total land has been utilized for vegetative cover in the basin. Due to climate change and intensifying human activities, particularly increasing human withdrawal of water for agriculture irrigation, streamflow in the Yellow River has significantly decreased since 1980s (Li et al., 2017; Zhang et al., 2009).

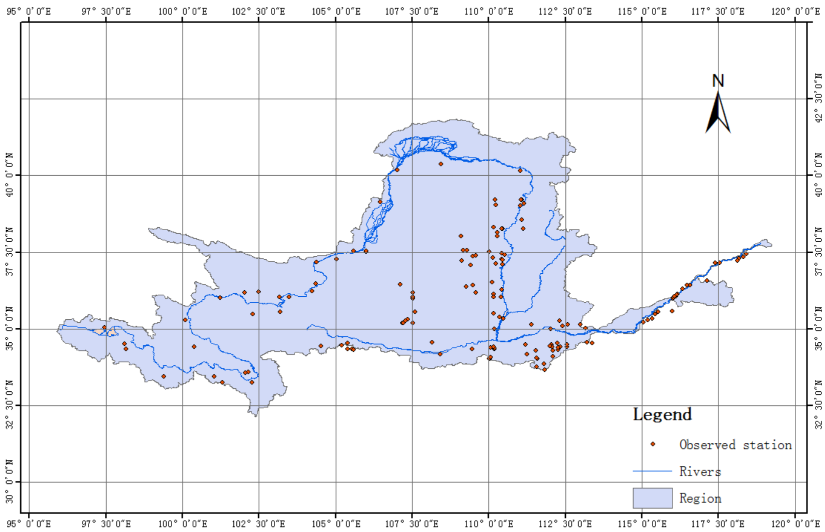

Figure 2. *Geographical boundary of Yellow River basin with grids distribution used for GCMs*

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GCM suitability assessment method

Taking the degree of fit of statistical values from GCM output and those from field-measured data as objective functions, the performance of each such function was scored, thereby evaluating the comprehensive performance of GCMs.

The mean value, coefficient of variability (= standard deviation / mean value), and normalized root mean square error (NRMSE) were used to characterize closeness between the mean value and deviation of GCM output variables and those of measured variables. NRMSE was defined as the ratio of root mean square error to the corresponding standard deviation.

Pearson correlation coefficients in both chronological (multiyear average monthly sequence) and spatial (mean value of climate elements at stations) sequences were used to characterize the closeness between GCM simulation and measured values, thereby evaluating the simulated variables' intraannual change and spatial degree of fit.

The rank-based nonparametric Mann-Kendall method was used to detect long-term tendencies (MK Zc) and magnitudes (MK Slope) of every variable. If areas where the MK Zc of GCM output variables matched measured values did not reach 95%, MK Slope was not included in the scoring.

Empirical orthogonal functions (EOFs) were used to qualitatively and quantitatively characterize variable spatiotemporal variation. Preliminary analysis showed that the first and second EOF characteristic vectors of nearly 20 variables were able to explain most of the deviations. Hence, these two vectors were included in rank scoring.

Two statistics of the probability density function, Brier score (BS) and skill score (Sscore), were used to evaluate the effectiveness of GCM simulation of that function. BS is the mean square deviation of probabilistic prediction, and Sscore is used to describe the overlap between the computed and measured probability distribution.

A total of 11 statistics were included, i.e., mean, coefficient of variability, NRMSE, temporal correlation coefficient, spatial correlation coefficient, trend analysis rank statistics, trend analysis variation magnitude, first and second EOF characteristic vectors, and BS and Sscore of the probability density function. Performance of each of the statistics was considered as one of the objective functions. A multi-criteria rank score (RS) value was computed for every objective function by assigning a value of 0-10, based on GCM performance. The calculation was:

$$RS_i = \frac{x_i - x_{\min}}{x_{\max} - x_{\min}} * 10, x_i < x_{\max} \quad \text{(Eq.1)}$$

where x_i is relative error in a statistic between simulated and measured values. The smaller the value is, the lower the score is. For a specific GCM output variable, the final total score was calculated by averaging the scores of all statistics. The better the simulation results, the lower the score. The score represents the degree of fit between GCMs outputs and measured values. It could be used to compare and analyze different GCMs, but it does not indicate the actual simulation accuracy of a given GCM.

Downscaling model

The Statistical Downscaling Model (SDSM) has both of deterministic transfer functions and stochastic components (Wilby et al., 2002). It has been widely applied in the statistical downscaling studies for both climate variables and air quality variables, and

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has been recommended by the Canadian Climate Impacts and Scenarios (CCIS) project (http://www.cics.uvic.ca).

An automated regression-based statistical downscaling model (ASD) (Hessami et al., 2008), inspired by the existing SDSM was developed within the Matlab environment, which is an easy to use graphical user interface for the statistical downscaling of GCM outputs to regional or local variables and already been successfully applied in major river basins of the east monsoon region in China (Xu et al., 2013).

Distributed hydrological models

The Taihu basin, located in the Yangtze River Delta region, is selected as the typical area for hydrological modeling in this study, which is characterized by a flat terrain, a complex river network, and various polder areas, without a clear outlet. Based on a 90-m DEM, it is difficult to extract the basin river network and simulate runoff yield and concentration over the entire basin. Therefore, the distributed Variable Infiltration Capacity (VIC) model based on grids was used to directly obtain gridded runoff depth in the basin. The VIC model considers physical exchange processes of atmosphere-vegetation-soil, primarily reflected by the variation of water and heat conditions and water and heat transfer in those three components. The model has been widely used to study the effects of climate change on hydrologic processes (Yuan et al., 2005; Liu, 2004; Su and Xie, 2003; Lohmann et al., 1998; Nijssen et al., 1997; Abdulla et al., 1996). VIC can either simultaneously simulate atmospheric-hydrological energy balance and water balance, or just calculate water balance, export runoff depth, and evaporation at each grid. Through a runoff concentration model, it transforms grid runoff depth into water flow at the basin outlet, eliminating the shortcomings of traditional hydrological models in the description of thermal processes.

The upper reaches of the Yellow River basin were selected to investigate the influences of climate change on the basin water cycle in this study. The reasons for this selection were: (1) The upper reaches are the origin of the Yellow River, and runoff variation there affects the entire basin; (2) future runoff change in the upper reaches can be used to validate basin-wide runoff change; (3) results of sub-basin estimation can provide accurate and detailed information for future runoff variation tendencies. In the upper reaches of the Yellow River basin, there is strong terrain fluctuation, an extractable river network, and abundant hydrologic data for the basin outlet. The Soil and Water Assessment Tool (SWAT) model (Arnold et al., 1998; Neitsch et al., 2005) was used for distributed hydrological simulation. Parameter sensitivity was analyzed by use of the built-in sensitivity analysis module.

Dataset

To include as many GCMs as possible from different countries and ensure the integrity and reliability of GCM outputs, 23 GCMs recommended by the IPCC were selected (*Table 1*). Monthly series including 15 climate variables, i.e., average temperature, relative humidity, longitudinal and latitudinal wind speeds, geopotential heights at the 500, 700 and 850 hPa levels, and two surface climate variables (temperature and precipitation) were used in this study. All data were from the IPCC data center. Details of the GCMs are on the website http://ipcc-ddc.cru.uea.ac.uk. All GCM output data were normalized to 2.5° × 2.5° by interpolation. Data series were from the period 1950 to 1999/2000.

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Table 1. *Information on GCMs*

GCMs	Abbreviation	Developing research institute	Country	Resolution	Period
BCCR:BCM20	BCCR	Bjerknes Centre for Climate Research	Norway	1.9° × 1.9°	1961-2000
PCM	PCM	National Center for Atmospheric Research	United States	2.8° × 2.8°	1961-1999
CCSM3	CCSM3	National Center for Atmospheric Research	United States	1.4° × 1.4°	1961-2000
CGCM2.3.2	MRI	Meteorological Research Institute	Japan	2.8° × 2.8°	1961-2000
CGCM3.1_T47	CGCM47	Canadian Centre for Climate Modelling and Analysis	Canada	2.8° × 2.8°	1961-2000
CGCM3.1_T63	CGCM63			1.9° × 1.9°	
CNRM:CM3	CNRM	National Centre for Meteorological Research	France	1.9° × 1.9°	1961-1999
CSIRO:MK30	CSIRO30	Atmosphere Research, Commonwealth Scientific and Industrial Research Organization	Australia	1.9° × 1.9°	1961-2000
CSIRO:MK35	CSIRO35			1.9° × 1.9°	
ECHAM4	ECHAM4	Meteorological Research Center	Germany	2.8° × 2.8°	1961-2000
ECHAM5	ECHAM5	Max Planck Institute für Meteorologie		1.9° × 1.9°	1961-2000
FGOALS:g10	FGOALS	State Key Laboratory of Numerical Modeling for Atmospheric Sciences and Geophysical Fluid Dynamics (LASG) / Institute of Atmospheric Physics	China	2.8° × 2.8°	1961-1999
GFDL:CM20	GFDL20	U.S. Department of Commerce / National Oceanic and Atmospheric Administration / Geophysical Fluid Dynamics Laboratory	United States	2.0° × 2.5°	1961-2000
GFDL:CM21	GFDL21			2.0° × 2.5°	
GISS:AOM	GISSAOM	National Aeronautics and Space Administration (NASA) / Goddard Institute for Space Studies (GISS)	United States	3° × 4°	1961-2000
GISS:EH	GISSEH			4° × 5°	
GISS:ER	GISSER			4° × 5°	
HadCM3	HadCM3	Met Office Hadley Centre for Climate Science and Services	United Kingdom	2.5° × 3.75°	1961-1999
HadGEM1	HadGEM1			1.3° × 1.9°	
INM:CM30	INM	College of Computational Mathematics	Russia	4° × 5°	1961-2000
IPSL:CM4	IPSL	Pierre-Simon marquis de Laplace	France	2.5° × 3.75°	1961-2000
MIROC3.2_hires	MIROC-h	Climate System Research Center, Tokyo University; National Environment Research Institute; Frontier Research Center for Global Change (JAMSTEC)	Japan	1.1° × 1.1°	1961-2000
MIROC3.2_medres	MIROC-m			2.8° × 2.8°	

ERA-40 and NCEP reanalysis data were taken as the measured data of the meteorological variables to assess the suitability of GCM outputs. Gridded data of monthly average temperature and monthly precipitation from the China Meteorological Administration (http://ncc.cma.gov.cn) were used, which were resampled to 2.5° × 2.5° grids.

Results and Discussion

GCM suitability assessment results

The Yangtze River basin

Comprehensive assessment results indicate that FGOALS, ECHAM4, ECHAM5, HadCM3, HadGEM1 and MRI had better performance in the Yangtze River basin than other GCMs (*Fig. 3*). Zhang et al. (2005) investigated spatiotemporal and circulation characteristics of the water vapor budget in the basin, and emphasized the influence of latitudinal water vapor transport originating from the Bay of Bengal on basin relative humidity. The simulation results of longitudinal and latitudinal wind speed strongly affected the spatiotemporal distribution of basin relative humidity. In the present study, four GCMs that effectively simulated relative humidity (ECHAM4, FGOALS, HadCM3 and HadGEM1) also showed good simulation of longitudinal and latitudinal wind speeds, consistent with previous studies. Temperature simulation results were better than other variables (including relative humidity), also demonstrated in previous studies. Xu et al. (2002) simulated climate change in East Asia based on five climate models, including temperature, precipitation, diurnal range and water vapor data. Their assessment results indicated that ECHAM4 and HadCM2 had best performance in China. In the present study, these two models also demonstrated promising outcomes. However, during

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assessment of GCM suitability in the Murray-Darling Basin of Australia, Maxino et al. (2008) obtained larger GCM-computed values of temperature than measured values for most GCMs. In contrast, in an assessment of GCM suitability in the Yellow River basin by Cao et al. (2009) the computed temperature was consistently cooler. In the present study, the computed temperature was warmer in most GCMs than measured. This might have resulted from a large difference of regional climate and circulation characteristics in the basins, as well as different GCM responses to climate characteristic variation (Xu et al., 2015; Miao et al., 2016). Therefore, it is necessary to assess GCM suitability across different basins.

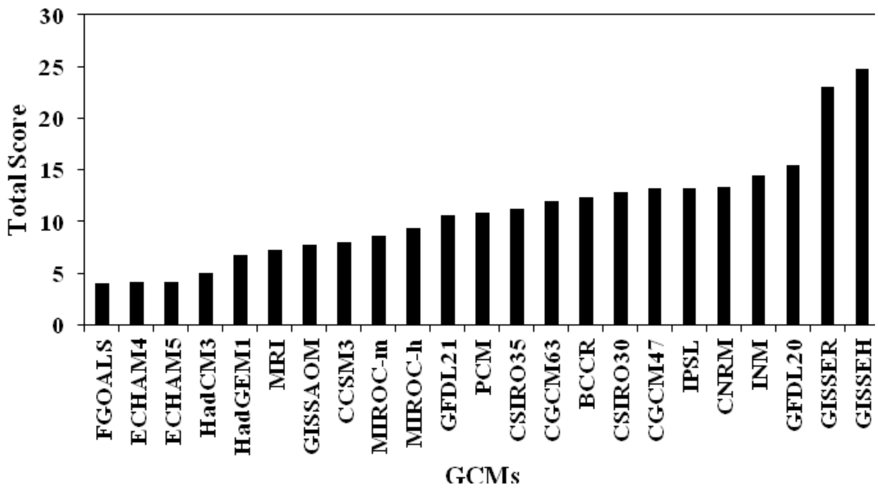

Figure 3. *Comprehensive scoring of GCMs in Yangtze River basin*

Specifically, we chose the Taihu basin to investigate the influences of climate change on the water cycle in the Yangtze River basin and evaluate GCM suitability. This basin is in the southern Yangtze River Delta. Associated administrative regions include southern Jiangsu, Jiaxing, Huzhou, part of Hangzhou in Zhejiang, and most of Shanghai, one of the most economically developed and populated regions in China. The basin water cycle is extremely sensitive to climate change.

Fig. 4 shows RS scores in increasing order. The lower the score is, the better the GCM performance for climate simulation is. Thus, it is seen that the BCCR model had the best performance overall. Therefore, it was used to assess the impacts of climate change. The simulated daily climate data were exported to the A1B scenario including two components, the current period (1961-2000, 20c3m scenario) and future periods (2046-2065 and 2081-2100). A1B is characterized by balanced economic development and greenhouse gas emissions, and is therefore suitable for future planning of coordinated water-economic-environment development in the Taihu basin.

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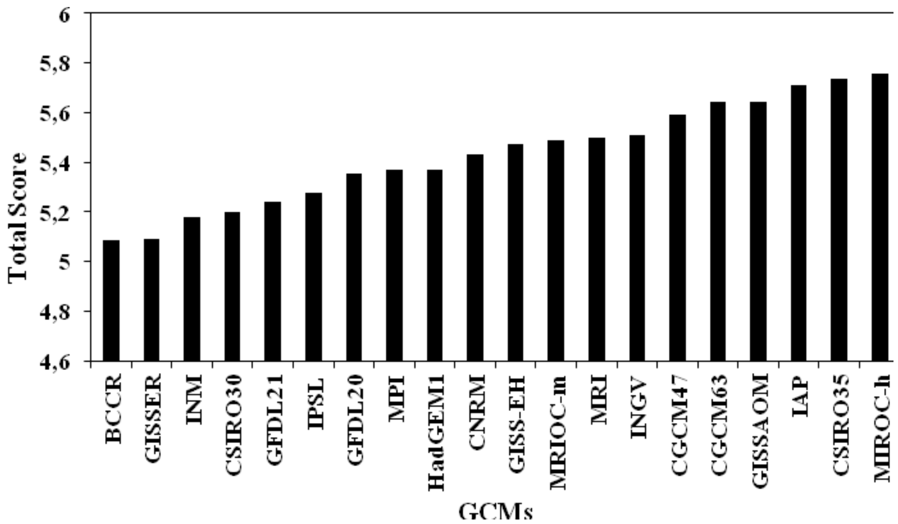

Figure 4. *Comprehensive scoring of GCMs in Taihu basin*

The Yellow River basin

25 grids of GCM data over the Yellow River basin were selected (*Fig. 2*). All GCM data were interpolated to a resolution of 2.5° × 2.5°. The comprehensive results showed that the top ten climate models were the MRI, HadGEM1, INM, CSIRO30, MIROC-M, HadCM3, BCCR, GFDL20, CGCM47, and GFDL21 (*Fig. 5*).

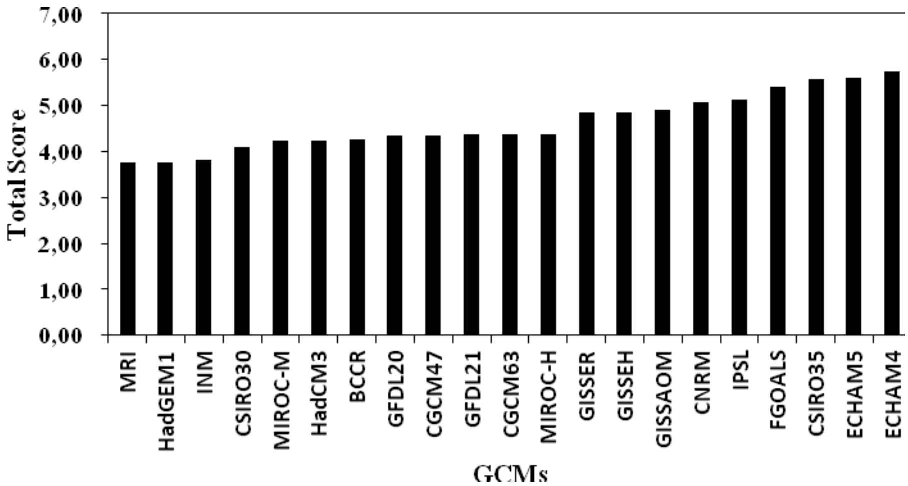

Figure 5. *Comprehensive scoring of GCMs in Yellow River basin*

While studying summer water vapor transport in the Asian monsoon zone and effects on precipitation in China, Zhou et al. (2008) observed the important role of longitudinal and latitudinal water vapor transport by the Indian monsoon to relative humidity of the basin in China. Also, it was reported that relative humidity was to a large extent affected by the simulation performance of longitudinal and latitudinal wind speeds. In the present study, the GCMs with favorable simulation of relative humidity (MRI, HadGEM1 and

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GFDL20) also showed good performance in simulation of those wind speeds. This is consistent with the finds of Zhou et al. (2008).

To analyze uncertainty caused by multiple GCMs and different scenarios and comprehensively consider the consistency and integrity of scenario data in different models, we compared A2 and B1 scenario data in MRI, CSIRO30 and INM, which had the best performance in the Yellow River basin.

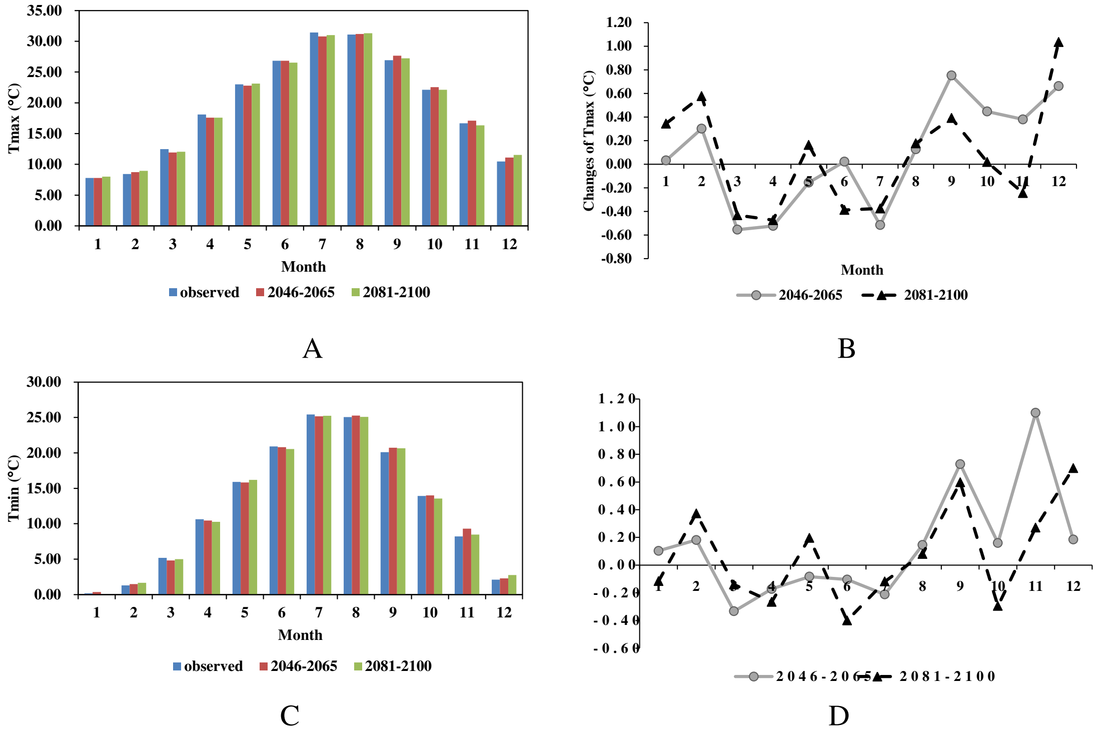
Impacts of climate change on water cycle

The Yangtze River basin

Construction of future climate change scenario

In a previous study by the authors (Chu et al., 2012), we conducted a comparison of statistical downscaling models for the Taihu basin. Specifically, the SDSM and ASD model were used to construct future climate change scenarios for that basin. The results indicated that ASD optimized the selection of predictors, and the simulation results were superior to those of SDSM. Therefore, the ASD model was used herein to construct future climate change scenarios.

ASD downscaling results revealed that the variation of future precipitation was complex and substantial compared to that of future temperature. *Fig. 6* shows that in the two future periods (2046-2065 and 2081-2100), the majority of months showed an increasing tendency in precipitation. The increase was large in spring and summer (except August), but smaller in other months. Compared to precipitation in 1961-1990, precipitation ranged between −0.3 and 83.6 mm and −5.3 and 89.5 mm in 2046-2065 and 2081-2100, respectively. In general, precipitation in the two future periods will not change significantly. Intra-annual variation was consistent, with reduced precipitation in January, August and December, and increased precipitation in March, May and July.



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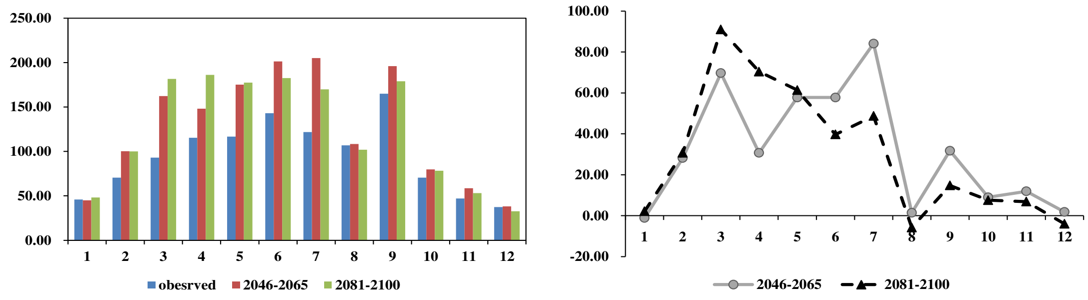

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Figure 6. *Daily maximum temperature (A-B), minimum temperature (C-D), precipitation (E-F) and their variations in the Taihu basin during two future periods predicted by ASD downscaling model (A, C, and E are comparisons between three elements and their measured values; B, D, and F are monthly changes compared to baseline period)*

Response to climate change of water cycle

The authors successfully constructed VIC for the Taihu basin (Liu et al., 2010), which was used in the present study. For meteorological data series under the A1B scenario during the baseline period (1961-1990) generated by the ASD model, including daily precipitation, daily maximum and minimum temperatures, the Thiessen polygon method was used to interpolate the data to 5 km × 5 km grids, thereby creating climate forcing data. Based on soil and vegetation parameters of the constructed VIC model of the Taihu basin (Liu et al., 2010), the model was run on 1452 grids of the basin, exporting daily runoff depth data at every grid for the period 1961-1990.

Similarly, based on meteorological data series in the future periods (2046-2065 and 2081-2100) generated by ASD, including daily precipitation, daily maximum and daily minimum temperatures, daily runoff depth data at every grid were exported.

Fig. 7 shows monthly changes of runoff depth on each grid for 2046-2065, compared to the baseline period. It is seen that in most areas of the basin, there was a decreasing tendency in monthly runoff depth in the future, of varying magnitude. Areas with large reductions of runoff depth were western Zhejiang and Zhangzhou-Jiaxing-Huzhou; the other areas showed lesser reductions. Particularly, Shanghai showed an increasing tendency during most of the year (January-April and September-December). Western Zhejiang demonstrated a slight increase in runoff depth during flood season.

Fig. 8 shows monthly changes of runoff depth at every grid between 2081-2100 and the baseline period. It is seen that in most areas of the basin, there was a decreasing tendency of monthly runoff depth in the future, with varying magnitude. The runoff depth in the western Zhejiang area tended to decrease compared to the baseline period, but the magnitude of decrease was less than that during 2046-2065. The Yangchengdian area had the largest reduction in runoff depth during March and May, whereas the Hangzhou-Jiaxing-Huzhou area showed a relatively large reduction in winter. Similar to the earlier period of 2046-2065, there was an increasing tendency in Shanghai during most of the year (January-April and October-December).

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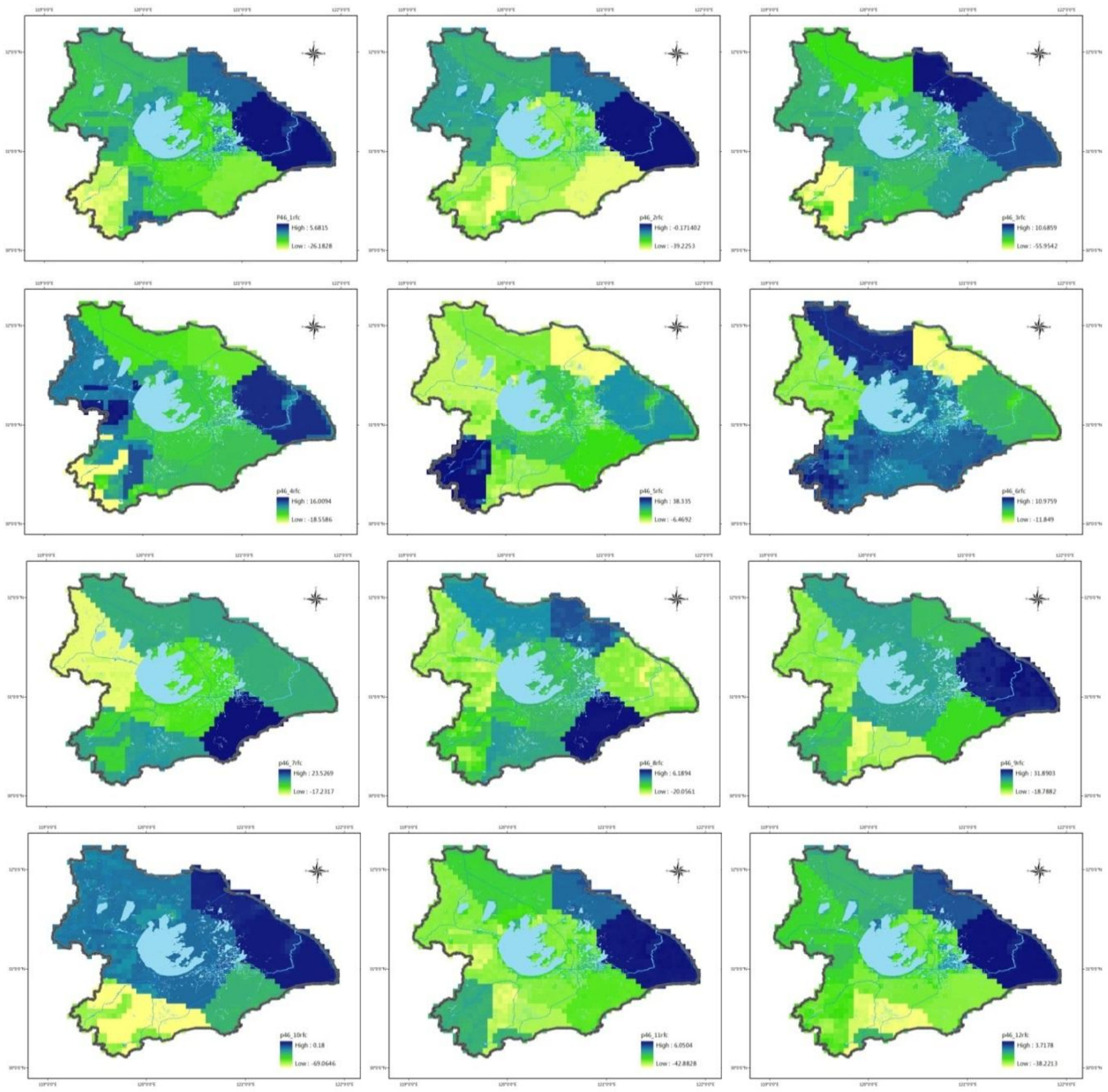

Figure 7. *Spatial distribution of monthly runoff depth variation during 2046-2065 under A1B scenario*

The Yellow River basin

Construction of future climate change scenario

Based on results of GCM suitability assessment, output data of three GCMs (MRI, CSIRO30, and INM) in the A2 and B1 scenarios were used and underwent interpolation to form 2.5° × 2.5° grids. The three simulation periods were the same as above. The SDSM model was used for downscaling. Twelve daily predictors were chosen, covering 25 grids in the Yellow River basin. The observation data contained daily precipitation, average temperature, and maximum and minimum temperatures at 79 stations in the basin from 1961 to 1990.

Fig. 9 displays the simulation results of monthly maximum and minimum temperatures from six conditions (three GCMs, each using the A2 and B1 scenarios). It is

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seen that both monthly maximum and minimum temperatures had increasing tendencies in most months of the year for all six conditions.

The variation of precipitation was substantially different between conditions (*Fig. 10*). Overall, the CSIRO30 model showed a minimal reduction (monthly variation < 25%) in precipitation, whereas MRI produced the greatest precipitation increase.

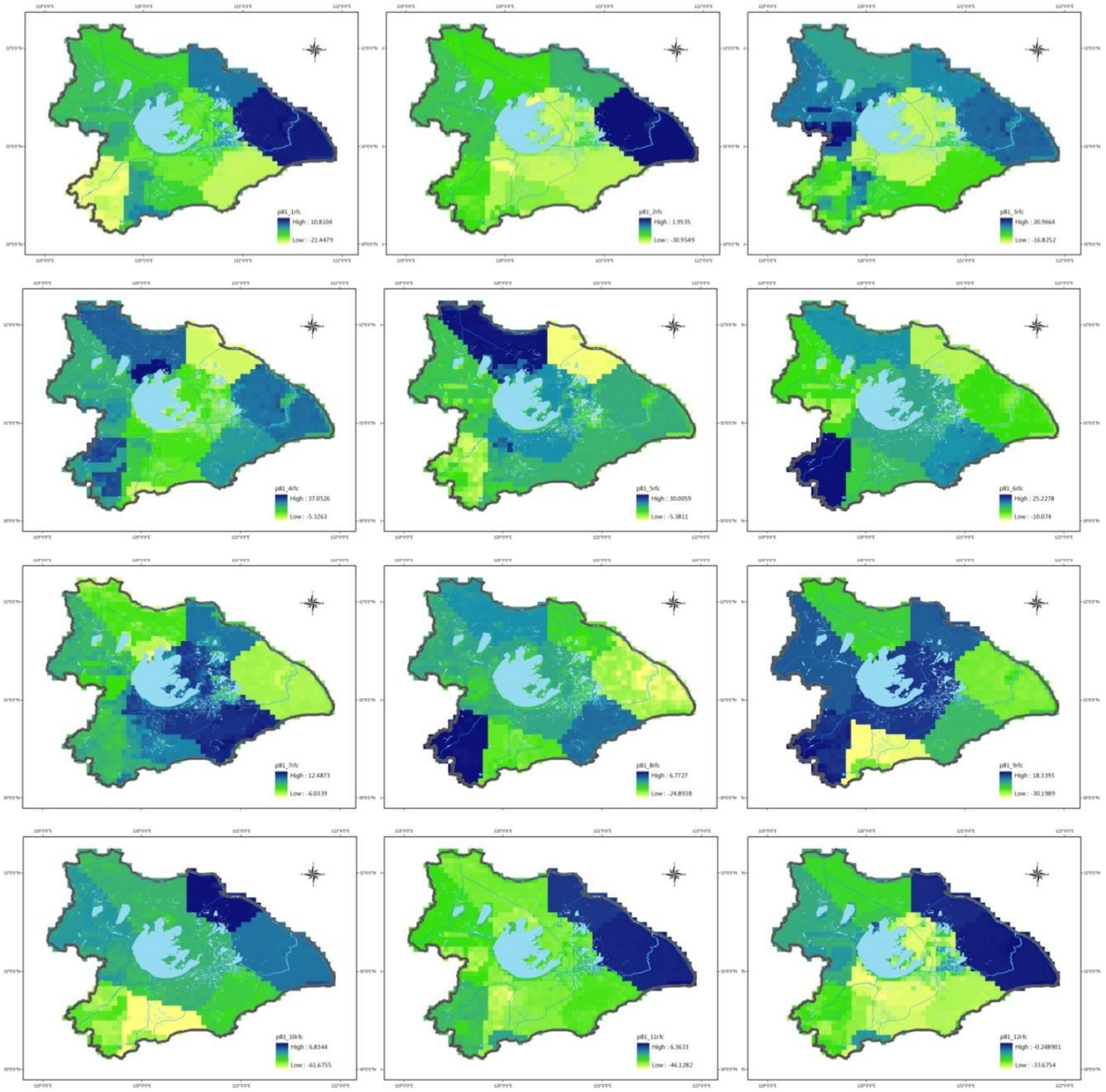
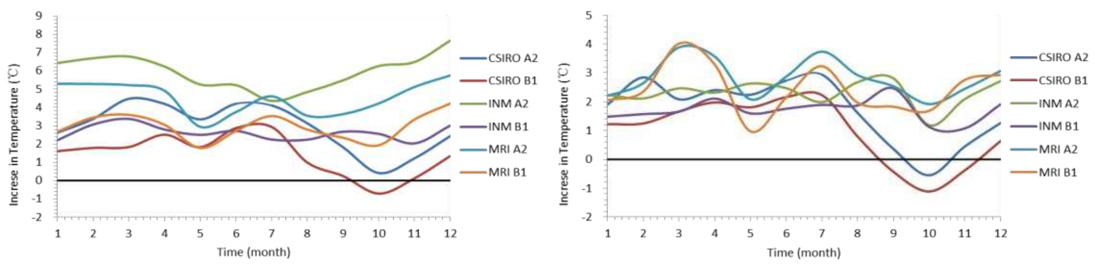


Figure 8. *Spatial distribution of monthly runoff depth variation during 2081-2100 under A1B scenario*



A B

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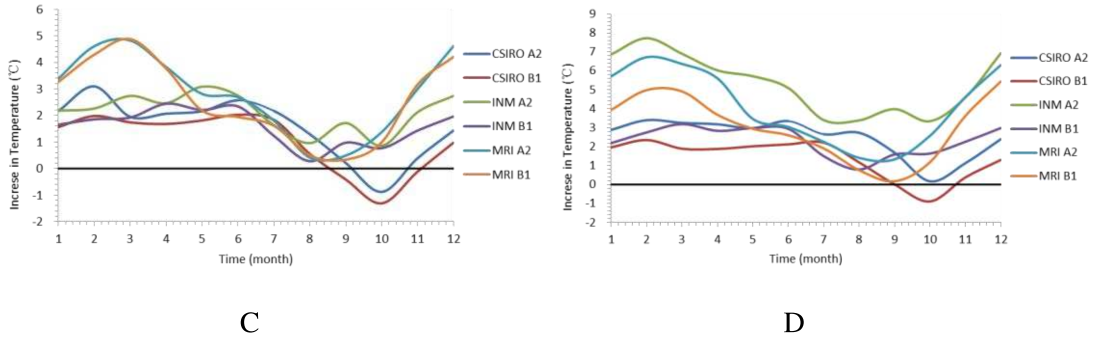

Figure 9. *Changes of simulated monthly maximum (A and B) and minimum (C and D) temperature under scenarios A2 and B1 during the period of 2046-2065 and 2081-2100 respectively*

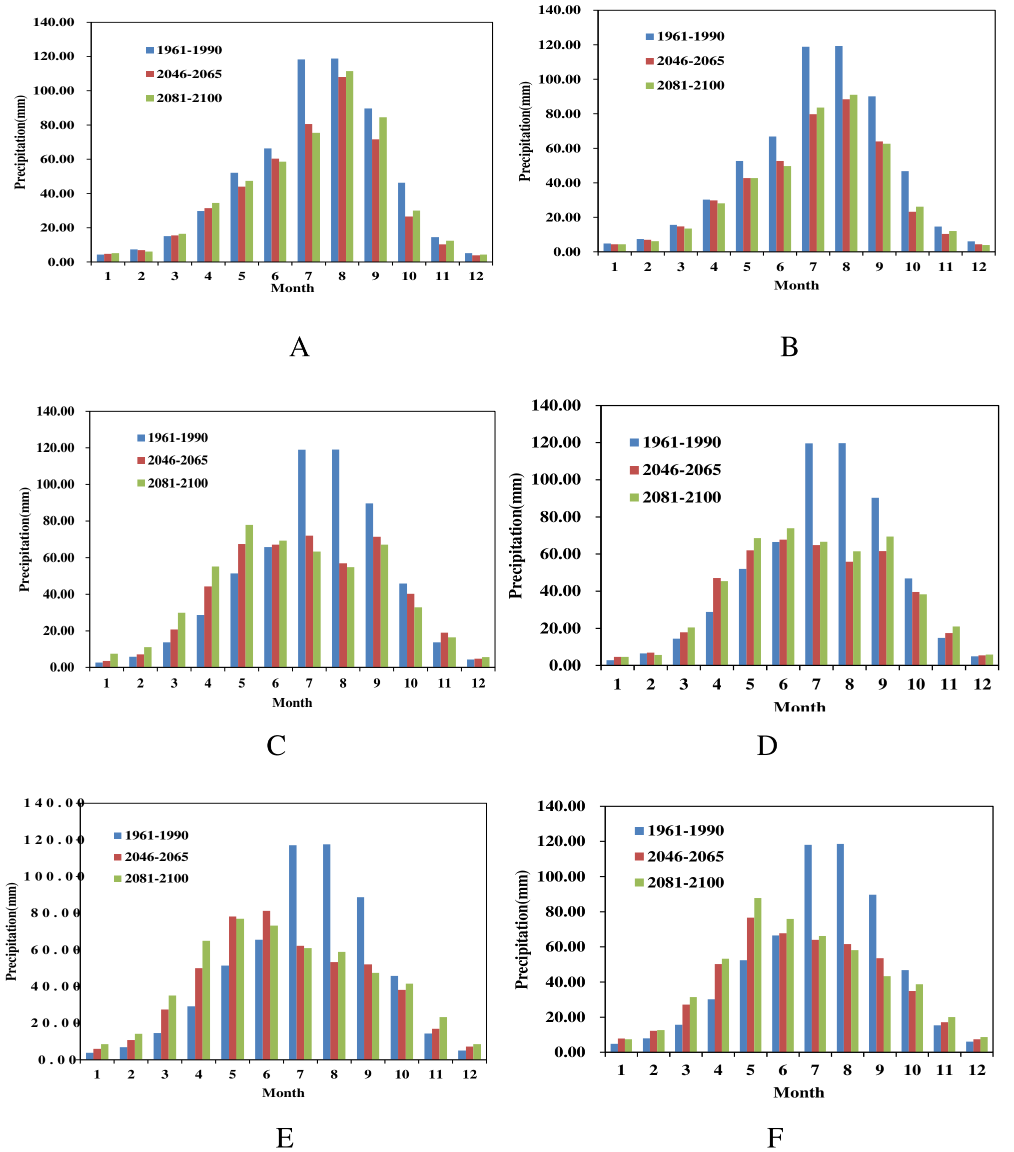

Figure 10. *Simulation results of precipitation from three GCMs (A-B: CSIRO30; C-D: INM; E-F: MRI) under various scenarios (a, c, and e: A2; b, d and f: B1)*

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Response to climate change of water cycle

(1) Construction of distributed hydrological model

The upper reaches of the Yellow River are on the Qinghai-Tibetan Plateau, bordering the Bayan Har Mountains in the south, Qaidam Basin in the north, Kunlun mountains in the west, and Loess Plateau in the east. The geomorphology is alpine grassland. The segment above Lanzhou (hereafter referred to as the upper reaches) has a total length of 2119 km and catchment area of 224,749 km^2, accounting for 28% of that in the entire basin. *Fig. 11* shows the location of the river basin.

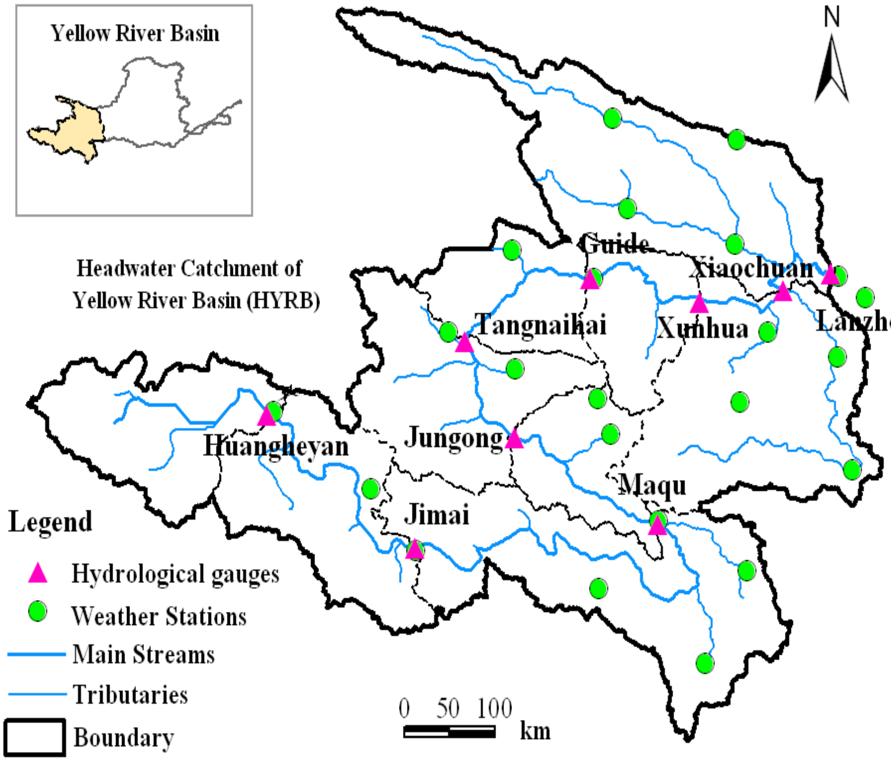

Figure 11. *Upper reaches of Yellow River*

The SWAT model with the built-in parameter sensitivity analysis module was calibrated and validated during 1971-1985 and 1986-2000, respectively. *Fig. 12* shows a comparison between discharge simulation and measured values at the Lanzhou hydrological station during the calibration and validation periods. In general, model indexes were satisfactory. The Nash-Sutcliffe efficiency and determination coefficients were 0.667 and 0.773 in the calibration period, and 0.626 and 0.709 in the validation period. Hence, the model showed good performance in simulating water balance in the basin.

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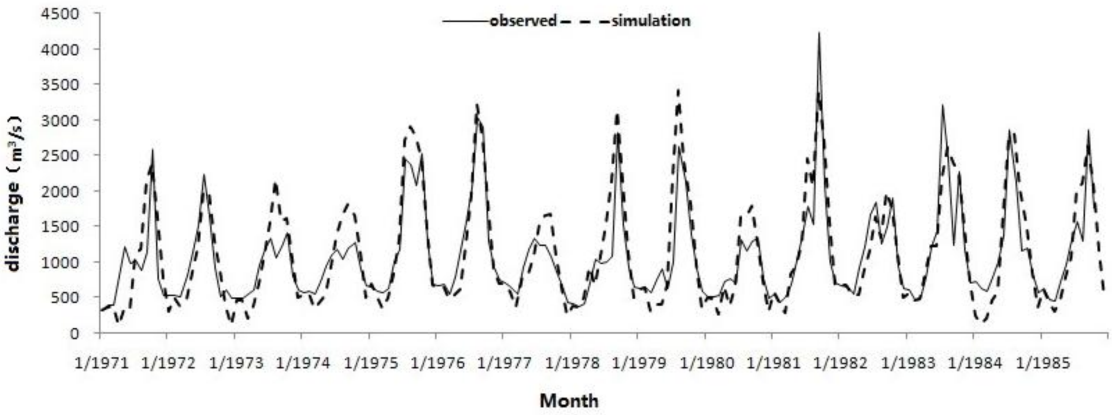
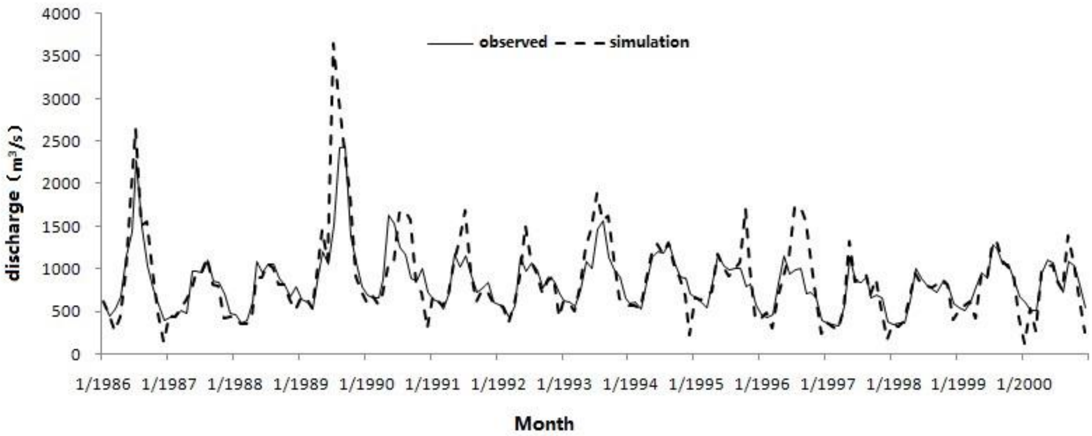


A



B

Figure 12. *Fitted curve of monthly discharge simulation to measured values at Lanzhou hydrological station (A: calibration period; B: validation period)*

(2) Response of runoff to future climate change in upper Yellow River basin

Figs. 13-15 present the spatial distribution of mean annual runoff depth during 2046-2065 and 2081-2100 compared to the baseline period, for every sub-basin and downscaling scenarios of the three GCMs. The runoff depth variation of the CSIRO model was -35.8 to 43 mm. In most areas, the variation was between −5 and −20 mm. Overall, the tendency of basin runoff was consistent between different scenario combinations, with runoff decreases in the majority of sub-basins. The area of runoff depth reduction was less under the B1 scenario than that under the A2 scenario. Runoff depth decreased greatly in the Hongyuan, Jiuzhi and Ruoergai areas, generally between −35 and −21 mm. In general, in the downscaling scenario of the CSIRO model, basin runoff depth tended to decrease in most sub-basins.

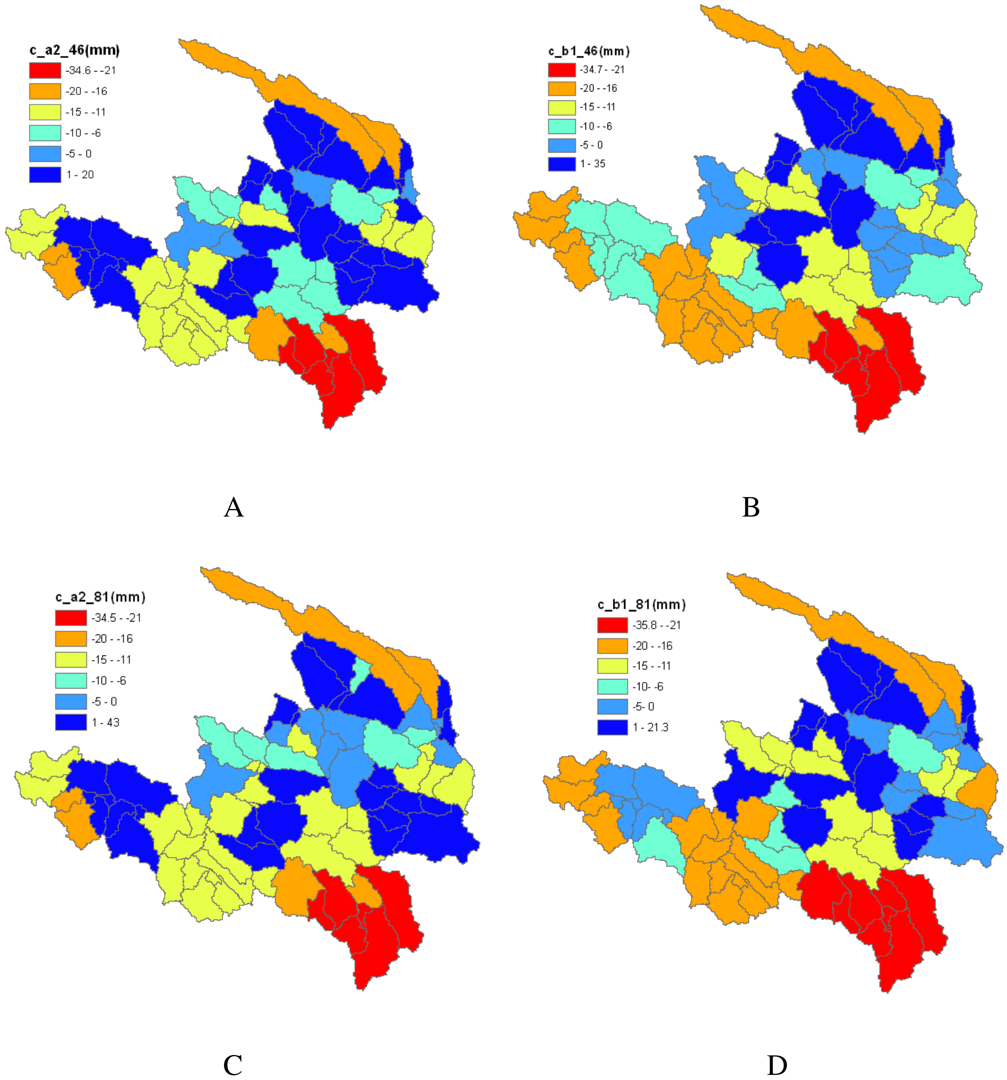


Figure 13. *CSIRO-estimated spatial distribution of mean annual runoff depth in sub-basins for 2046-2065 (A and B) and 2081-2100 (C and D) compared to baseline period*

For various scenario combinations of the INM model, changes of runoff depth were between −33.9 and 61 mm, with decreases in most sub-basins. The tendency of runoff depth with different scenario combinations was relatively consistent, showing decreasing tendencies (in excess of 20 mm) in southern sub-basins, and mixed increases and decreases in northern sub-basins. under the A2 emission scenario of INM, the area of sub-basins with reduced runoff during 2081-2100 was larger than that in 2046-2065. Under the B1 scenario, the area was smaller in 2081-2100. The Hongyuan, Jiuzhi, and Ruoergai areas had large reductions in runoff depth, between −31 and −21 mm.

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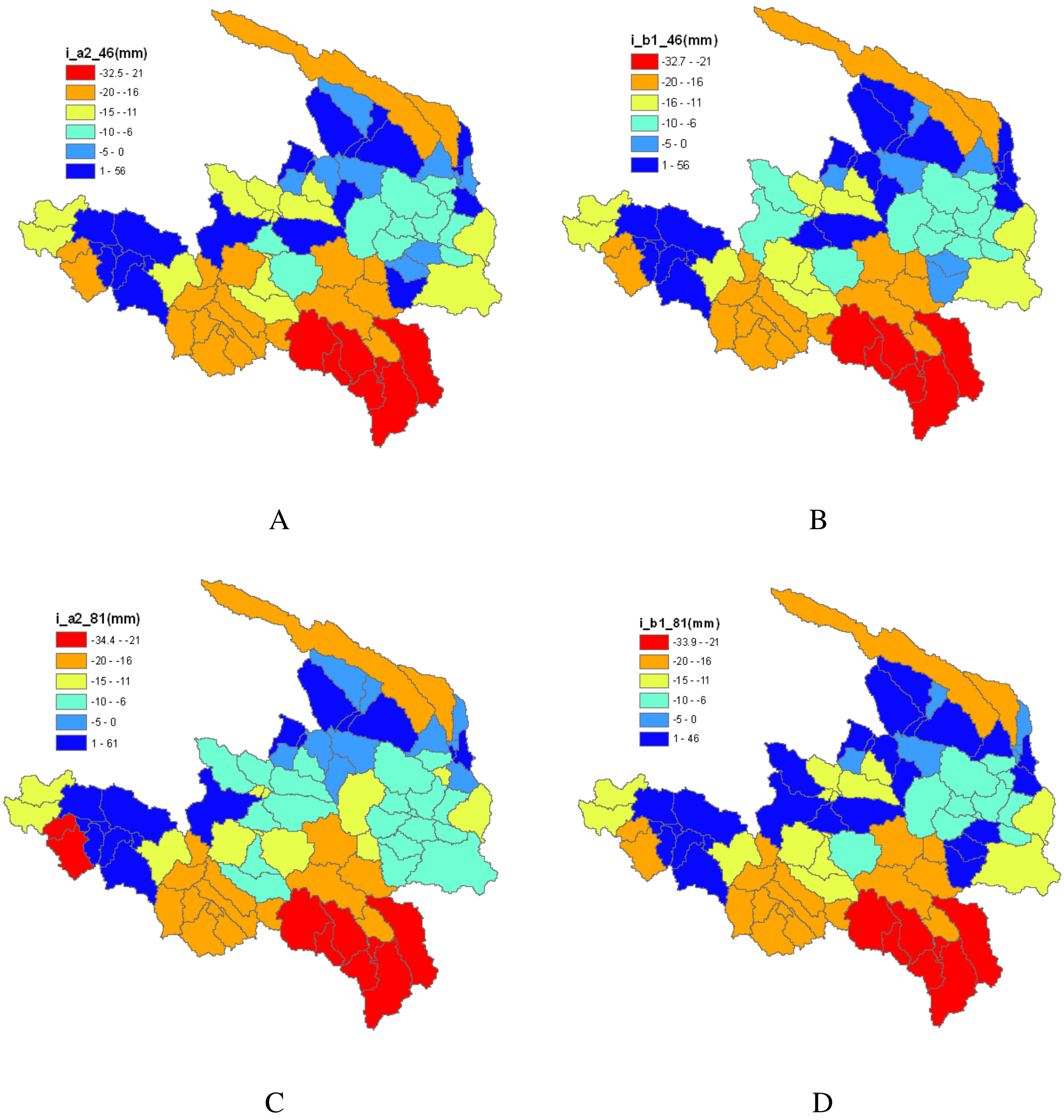

Figure 14. *INM-estimated spatial distribution of mean annual runoff depth in sub-basins for 2046-2065 (A and B) and 2081-2100 (C and D) compared to baseline period*

For different scenario combinations of the MRI model, runoff depth was between −32.9 and 69 mm, with over half the area of sub-basins showing a decreasing tendency in average runoff depth. In the period 2046-2065, the area of sub-basins with increasing runoff under the A2 scenario was larger than that under the B1 scenario, as was the area with reductions of 16-20 mm. For 2081-2100, the area of sub-basins with increasing runoff under the B1 scenario was larger than that under the A2 scenario, as was the area with reductions of 16-20 mm. The MRI model produced a decreasing tendency in runoff depth over half the basin area. In addition, that depth decreased greatly in Hongyuan, Jiuzhi and Ruoergai, generally between −35 and −21 mm.

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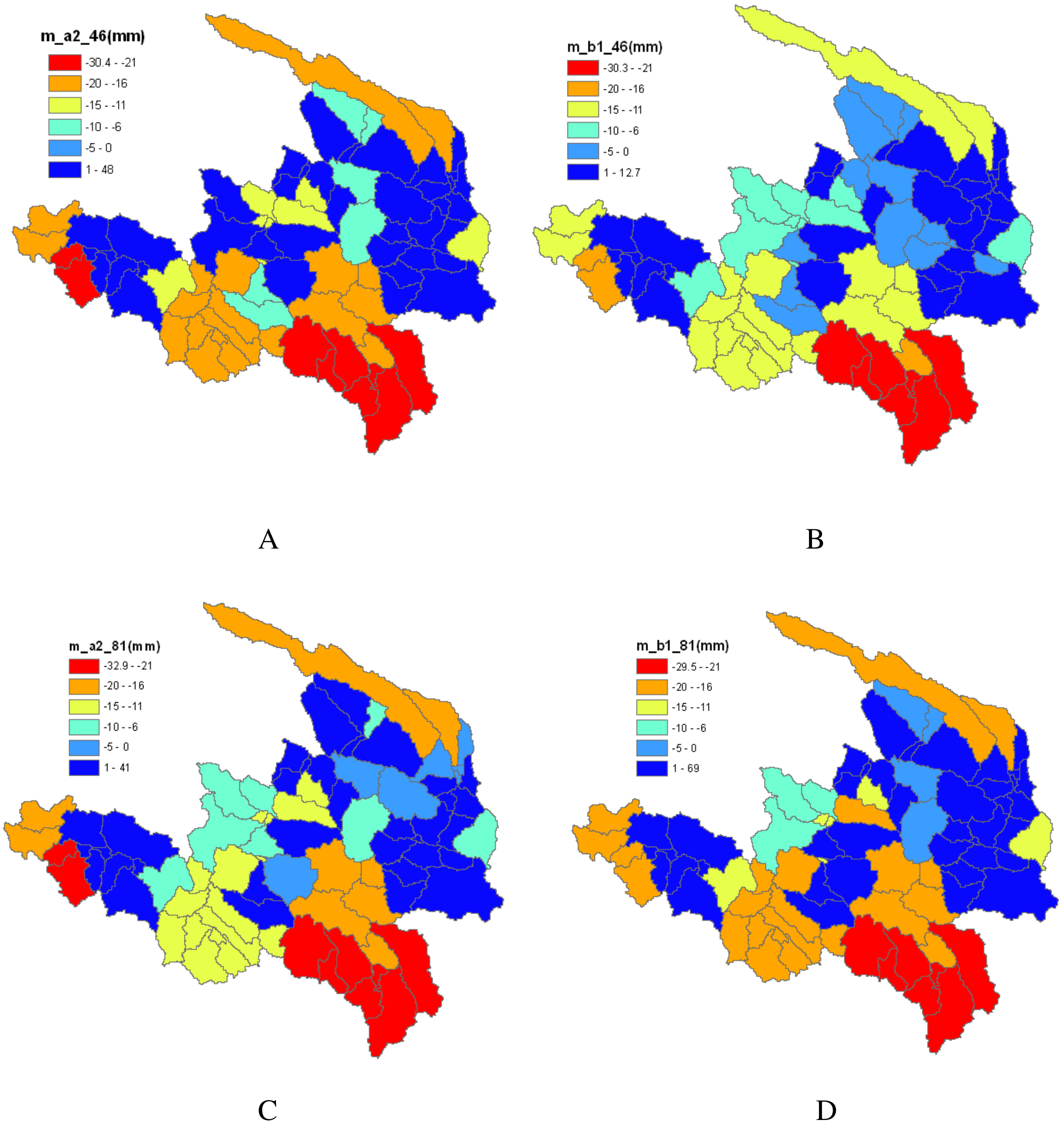

Figure 15. *MRI-estimated spatial distribution of mean annual runoff depth in sub-basins for 2046-2065 (A and B) and 2081-2100 (C and D) compared to baseline period*

Conclusions

As an important component of the climate system, the water cycle is extremely sensitive to climate change. In the present study, taking the typical areas of the Yangtze and Yellow River basins as objects, we conducted suitability assessments of GCMs and downscaling. Through coupling of GCMs and distributed hydrological models, the impacts of climate change on the river basin water cycle were quantified. The conclusions are as follows.

(1) Based on rank scoring, the degree of fit between statistical values of GCM output and those of reanalysis data was taken as the objective function. Scores were given according to the performance of every objective function, thereby obtaining comprehensive scores for the performance of each GCM in their simulation of the

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Yangtze and Yellow River basins. Specifically, the top three models were the MRI, HadGEM1 and INM for the Yangtze River, and FGOALS, ECHAM4 and ECHAM5 for the Yellow River. This indicates a substantial difference between the southern and northern regions of China with respect to the effects of climate change.

(2) Future precipitation and temperature data under the A1B scenario were generated by the ASD downscaling model, which were used to drive the VIC model on 5 km × 5 km grids. Simulation of hydrologic processes in the Taihu basin on the lower reaches of the Yangtze River showed that areas with substantial reduction in runoff depth during 2046-2065 compared to a baseline period were western Zhejiang and Hangzhou-Jiaxing-Huzhou. The western Zhejiang area showed a slight increase in runoff depth during flood season. Runoff depth in western Zhejiang tended to decrease in 2081-2100 compared to the baseline period, but this decrease was less than that during 2046-2065.

(3) The SWAT model gave relatively promising results for the upper reaches of the Yellow River basin. Under the A2 and B1 scenarios, runoff tended to decrease in future periods. In particular, the decrease was greater in 2081-2100 than in 2046-2065. Under different scenario combinations, annual average runoff in 2046-2065 and 2081-2100 was 27.507 and 25.737 billion m^3, respectively, reductions of 16.9% and 22.2% compared to the baseline period. Under various scenario combinations, spatial runoff variation in the upper basins of the Yellow River was consistent. In most areas there was a decreasing tendency in runoff depth. Specifically, the decrease was largest in Hongyuan, Jiuzhi and Ruoergai, in excess of 20 mm. Maximum runoff decrease did not vary much with the scenario combination, and was about −30 mm. However, the magnitude of increase was substantially different, between 12.7 and 69 mm.

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EVALUATION OF SOME MANAGEMENT STRATEGIES IN EUTROPHIC MOGAN LAKE, TURKEY: PHOSPHORUS MOBILITY IN THE SEDIMENT-WATER INTERFACE

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Abstract. Mogan Lake, a popular recreation area, is under the pressure of intense urban-industrial pollution, therefore some management strategies included occasional macrophyte harvesting, diverting running water into the lake to accelerate the improvement of its water, and dredging have been in progressing since 1995. This study was conducted on sediment and overlying water samples collected in April, July and October 2015 and January 2016 from Mogan Lake. The outstanding data for this study is presented as: i) detection of the sediment chemical composition and estimation of sediment phosphorus mobility, ii) comparison of the current status of the lake's eutrophication regarding phosphorus mobility with data from earlier studies regarding the common station. The research data has revealed i) sediment TP concentrations ranged from 620.00 µgP gDW^{-1}, to 1047.50 µgP gDW^{-1} while TN concentrations were between 3250.00 µg gDW^{-1} and 6325.00 µg gDW^{-1}. Sediment TOC concentrations ranged between 58850.00 µg gDW^{-1} and 79675.00 µg gDW^{-1}. Sediment iron content and loss on ignition values were determined as 1.608-3.415 mg gDW^{-1} and 9.05-20.94 %, respectively. The rank order of phosphorus fractions were evaluated as Ca≈P>Org≈P>CO_3≈P>Fe+Al≈P. ii) the phosphorus release values from the sediment (0.1754 – 1.1249 mg P $m^{-2}d^{-1}$) were higher than the earlier studies findings. Phosphorus release in the lake has increased over the past ten years but still remains at a generally low level together with some management activities in Mogan Lake, therefore, instead of the internal P source future research should focus on external sources. In shallow eutrophic systems such as Mogan Lake, sediments having phosphorus accumulation depends on deep interactions with principally antropogenic external sources. In conclusion the primary goal is to determine the adsorption capacity of the sediment in order to estimate the critical internal phosphorus load and the second goal should be to apply a sustainable monitoring program.
Keywords: *freshwater, phosphorus speciation, eutrophication, lake management, nutrient cycle*

Introduction

The primary gauge of the phosphorus cycle in natural freshwater bodies is the shift of phosphorus between the sediment and the overlying water. Better understanding of the occurrence of water phosphorus (P) at the water-sediment interface is vital to clarify P sources of origin in freshwater shallow lake ecosystems (Pu et al., 2017). Rather than focusing on total phosphorus content when examining the relationship between sediment phosphorus and eutrophication in a lake, it is more efficient to focus on the contents of the different phosphorus fractions (Christophoridis and Fytianos, 2006). The critical roles of P fractionation as regulators were manifested to interpret immediate and hysteretic internal liberation and indirectly represent anthropogenic inputs (Pu et al., 2017). In determining the sediment's role as either a trap or a source of phosphorus, it is important to use chemical extractions to examine different chemical P fractions in the sediment such as labile P, reductant P, metal-bound P, occluded P and organic P (Smolders et al., 2006).

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The accumulation of phosphorus in lake sediment generally occurs during heavy loading cycles, and its release into the overlying water is seen prior to reductions in external loading (Jiang and Shen, 2006; Watson et al., 2016). Several physical, chemical and biological measures (biomanipulation, phosphate inactivation, rooted macrophyte harvesting and sediment dredging) are generally undertaken in lakes to control eutrophication caused by internal phosphorus loading, which can be triggered by a reduction of external phosphorus loading (Smolders et al., 2006; Madura and Goldyn, 2009).

Several studies (Pulatsü et al., 2008; Topçu and Pulatsü, 2008; Pulatsü and Topçu, 2009) have been undertaken regarding the eutrophication-sediment interaction in Mogan Lake, which is an important recreation area due to its proximity to Ankara. Mogan Lake was chosen in this study due to the fact that it has been heavily influenced by human actions ranging from agricultural and domestic to semi-industrial pollution sources. Furthermore, it is thought that the data collected from the study will be of importance for the management of the lake, and these data could also be useful in the management of similar aquatic systems.

The first measures aimed at reducing external pollution loads in this shallow, eutrophic lake, located in the Gölbaşı Special Protected Area and under the pressure of intense urban-industrial pollution, were begun in 1995. These management strategies included occasional macrophyte harvesting, diverting running water into the lake to accelerate the improvement of its water, and dredging. The aim of this study was to give an overview in April, July, and October 2015 and January 2016 following the implementation of these management strategies in Mogan Lake including i) detection of the sediment chemical composition (total organic carbon, loss on ignition, total iron, total nitrogen, total phosphorus and phosphorus fractions), and determination of sediment-sediment overlying water phosphorus mobility (sediment phosphorus release/retention) with effective factors in a view towards phosphorus control and ii) comparison of the current status of the lake's eutrophication regarding phosphorus mobility with some earlier study data (Topçu and Pulatsü, 2008; Pulatsü et al., 2009) considering the common station.

Materials and Method

Study site

Mogan Lake, located 20 km south of Ankara, Turkey (39°47′N 32°47′E), is an alluvial pond that is fed by more than five streams of varying size. The lake's outflow is into Eymir Lake through a wetland on Mogan's north side. Mogan Lake's surface area is 7.20 km^2, its mean depth is 2.80 m, its lake volume is 13.72 × 106 m^3, and its average theoretical flushing rate is 1.35 times per year (Boşgelmez et al., 2005). Due to the external phosphorus load resulting from detergents in waste water effluents and fertilizers in agricultural run-off from nearby areas, Mogan Lake is highly eutrophic (Fakıoğlu and Pulatsü, 2005). Because the bed of the eastern littoral area of Mogan Lake is covered by stone, two research stations were established near the lake's western shore, where the lake bed is more suitable for obtaining sediment samples. There were rooted surface plants in the areas surrounding the stations, and the western shore of the lake is affected by dense development and exposed to anthropogenic pollutants. The research area and location of the stations are shown in *Figure 1*.

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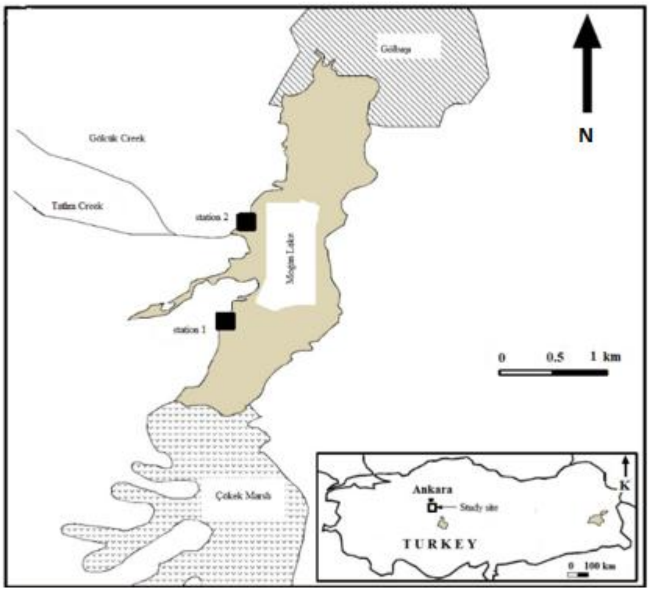

Figure 1. *Map of the lake Mogan with the sampling site (■)*

Field work

Three independent parallel sediment samples were collected from 70 cm of the littoral zone in the north-western part of the lake in April, July and October 2015 and January 2016 using Van Ween Grab. A sampling site deemed representative of the majority of the lake was selected. The sampling site was covered during the summer, primarily by *Chara vulgaris* and *Phragmites australis*, cattails (*Typha domingensis* Pers.), and two species of rush (*Juncus* spp.).

Lake water samples were collected from 0 to 10 cm above the sediment-water interface. The temperature, dissolved oxygen (DO) concentration and pH of the overlying water were measured onsite.

Analytical methods

Sediment samples and procedure

All samples were stored at 4°C before laboratory experiments and analyses. All analyses and extractions were done in triplicate, comprising the sediment composition determined for air-dried sediment samples homogenized by grinding (Kacar, 1995). The dried sediments (105°C, 24 h) were analysed spectrophotometrically for TP after digestion in a mixture of oxidizing acids. Sediment phosphorus fractionations were determined with the method of Hieltjes and Lijklema (1980), including (i) loosely sorbed phosphorus ($CO_3 \approx P$), extracted for 2 h with 1 M NH_4Cl, (ii) iron-plus-aluminium-bound phosphorus ($Fe + Al \approx P$), extracted for 17 h with 0.1 N NaOH, and (iii) calcium-bound phosphorus ($Ca \approx P$), extracted for 24 h with 0.5 N HCl. Organic-bound phosphorus ($Org \approx P$) was calculated as the difference between TP and the sum of the inorganic fractions. The sediment water content was determined by drying at 110°C for 16 h, and loss on ignition (LOI) was determined by the loss of weight during ignition at 550°C for 2 h. The total iron concentration was measured with Atomic Adsorption/Flame Emission Spectroscopy (APHA, 1995). Sediment total nitrogen was determined using the Dumas Method.

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All parameters were measured for all sections of the sediment and reported on a dry-weight basis. Concentration profiles were used to calculate the diffusive flux of soluble reactive phosphorus (SRP, given as P-release rates) towards the sediment surface, based on Fick's first law of diffusion (Shaw and Prepas, 1990). The concentrations of TP and soluble reactive phosphorus were measured according to the method outlined in APHA (1995).

Overlying water and sediment pore water analysis

Sediment pore water was obtained by vacuum filtration of the sediment samples (Eckert et al., 1997), and sediment overlying water was obtained by siphoning from the water 10 cm above the sediment. Water from these samples was analyzed in duplicate for soluble reactive P (SRP) (APHA, 1995).

Phosphorus release estimation

Potential phosphorus release from the sediment into the lake water by molecular diffusion was calculated by Berner (1980) (Shaw and Prepas, 1990):

$$Flux = \varphi . D . Q^{-2} . dc/dx . 86\,400 \qquad \text{(Eq.1)}$$

in which flux = SRP flux across the sediment-water interface (in mg $m^{-2}d^{-1}$)
φ = the water content by volume (dimensionless)
D = molecular diffusion coefficient (varies according to temperature)
Q^2 = tortuosity term (φ-0.8; (Shaw and Prepas, 1990))
dc/dx = the SRP gradient across the sediment-water interface (mg m^{-4}) 86 400 = the factor to convert s to d.

Statistical analyses

Statistical evaluation of data including some physiochemical properties of the sediment, determination of fractional phosphate concentrations, and analyses of the sediment overlying water were performed using the Windows Minitab and MStat databases according to the method established by Kesici and Kocabaş (2007).

Results

The differences between the months and stations with regard to the averages of total nitrogen, total organic carbon, total phosphorus, phosphorus fractions, total iron, organic matter concentrations and water contents found in the littoral sediment of Mogan Lake in April, July and October 2015 and January 2016 were found to be statistically significant ($p<0.05$) (*Table 1*, *Figure 2*). Information regarding the sediment composition at each of the Mogan Lake stations is shown in *Table 2*. The sediment composition at both stations was classified as rich in clay and loam (CL).

Differences between the months and stations with regard to the averages for water temperature, dissolved oxygen, pH, total filterable orthophosphate concentrations in the overlying water and SRP concentrations in the sediment pore water of Mogan Lake in the months of April, July and October 2015 and January 2016 were found to be statistically significant ($p<0.05$) (*Table 3*).

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Table 1. *Seasonal and spatial variations of sediment total nitrogen (TN), total organic carbon (TOC), total iron (TFe), Loss on ignition (LOI) and water content in Mogan Lake (Mean value±standard deviation, N=4)*

Properties	**Sample sites / Months**	**1**	**2**
TN ($\mu g\ gDW^{-1}$)	**April 2015**	$3350.00 \pm 125.83^{C\#a*}$	3250.00 ± 150.00^{Ca}
	July 2015	6250.00 ± 537.74^{Aa}	6325.00 ± 309.23^{Aa}
	October 2015	3975.00 ± 131.50^{Cb}	6175.00 ± 137.69^{Aa}
	January 2016	5175.00 ± 4.87^{Ba}	4150.00 ± 86.60^{Bb}
TOC ($\mu g\ gDW^{-1}$)	**April2015**	$63550.00 \pm 425.25^{BCa}$	$58850.00 \pm 2465.94^{Ba}$
	July 2015	$79675.00 \pm 5549.38^{ABa}$	$77700.00 \pm 3966.32^{Aa}$
	October 2015	$64750.00 \pm 3547.42^{BCa}$	$79250.00 \pm 1156.50^{Aa}$
	January 2016	$69600.00 \pm 248.33^{ABb}$	75550.00 ± 847.05^{Aa}
TFe ($mg\ gDW^{-1}$)	**April2015**	3.313 ± 0.040^{Aa}	1.608 ± 0.022^{Cb}
	July 2015	1.718 ± 0.111^{Bb}	3.415 ± 0.026^{Aa}
	October 2015	$1.913 \pm 0{,}030^{Bb}$	$3.238 \pm 0.010B^{a}$
	January 2016	3.098 ± 0.069^{Aa}	3.275 ± 0.051^{Ba}
LOI (%)	**April2015**	20.94 ± 1.41^{Aa}	$15.83 \pm 0.\ 08^{Ab}$
	July 2015	10.10 ± 0.13^{Ab}	9.05 ± 0.04^{Ba}
	October 2015	10.17 ± 0.77^{Bb}	15.54 ± 0.15^{Aa}
	January 2016	12.58 ± 0.22^{Ba}	11.88 ± 0.14^{Bb}
Water content (%)	**April2015**	88.25 ± 0.48^{Aa}	85.00 ± 0.41^{Ab}
	July 2015	76.50 ± 0.65^{Ba}	77.00 ± 0.41^{Ba}
	October 2015	87.50 ± 0.54^{Aa}	86.50 ± 1.19^{Aa}
	January 2016	88.25 ± 0.48^{Aa}	87.25 ± 1.10^{Aa}

[*] The different lower-case letters in the same row show the differences between stations ($p < 0.05$)
[#] The different upper-case letters in the same column show the differences between months ($p < 0.05$)

Table 2. *Spatial sediment grain size in Mogan Lake*

Properties		**Sample sites**	
		1st station	**2nd station**
Grain size (µm)	Clay (<4)	68.99	65.12
	Silt (4–63)	19.72	22.33
	Sand (63–500)	11.29	12.55
	Class	Clay+loam(CL)	Clay+loam (CL)

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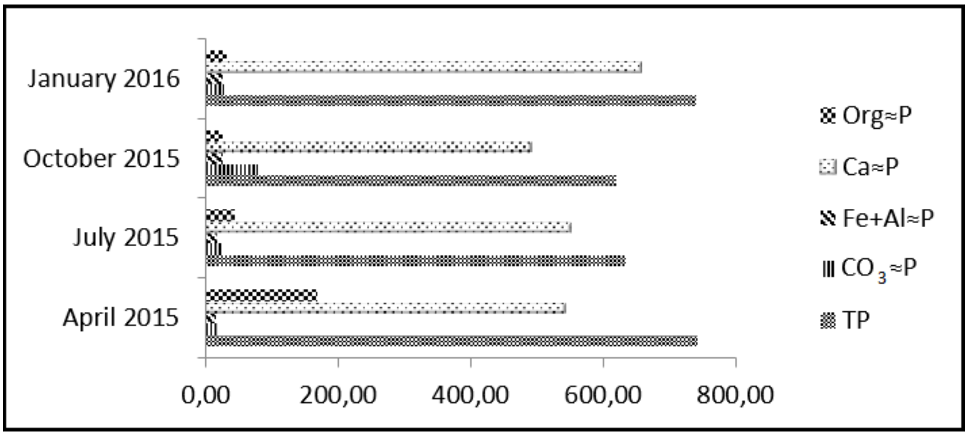
Table 3. *Seasonal and spatial variations of sediment overlying water's water temperature, dissolved oxygen, pH, and SRP with sediment porewater SRP in Mogan Lake (Mean value±standard deviation, N=4)*

Properties	Sample sites / Months	1	2
Water temperature (°C)	**April2015**	22.08±0.05$^{B\#a*}$	21.88±0.05^{Ba}
	July 2015	26.68±0.03^{Aa}	25.25±0.03^{Ab}
	October 2015	10.08±0.03^{Ca}	10.23±0.05^{Ca}
	January 2016	3.33±0.05^{Da}	3.48±0.03^{Da}
DO_2 (mg L^{-1})	**April2015**	9.88±0.23^{Bb}	13.45±0.10^{Ba}
	July 2015	11.20±0.11^{Ab}	15.72±0.02^{Aa}
	October 2015	6.31±0.00^{Ca}	5.90±0.01^{Db}
	January 2016	9.93±0.03^{Bb}	10.23±0.04^{Ca}
pH	**April2015**	9.42±0.01^{Ab}	9.56±0.02^{Aa}
	July 2015	8.88±0.01^{Bb}	9.46±0.05^{Aa}
	October 2015	7.99±0.00^{Db}	8.30±0.01^{Ba}
	January 2016	8.13±0.00^{Ca}	7.72±0.06^{Ca}
OW SRP (mg m^{-3})	**April2015**	39.68±0.79^{Bb}	80.16±0.79^{Aa}
	July 2015	40.48±0.79^{Ba}	17.46±0.92^{Cb}
	October 2015	65.08±0.91^{Aa}	38.89±2.71^{Bb}
	January 2016	62.70±7.82^{Aa}	78.57±3.27^{Aa}
PW SRP (mg m^{-3})	**April2015**	817.22±2.92^{Ba}	353.89±10.52^{Cb}
	July 2015	412.78±4.19^{Cb}	196.67±1.93^{Da}
	October 2015	836.11±7.39^{Ba}	809.45±1.06^{Aa}
	January 2016	960.56±11.24^{Aa}	411.67±8.72^{Bb}

*The different lower-case letters in the same row show the differences between stations ($p < 0.05$)
#The different upper-case letters in the same column show the differences between months ($p < 0.05$)

a)



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b)

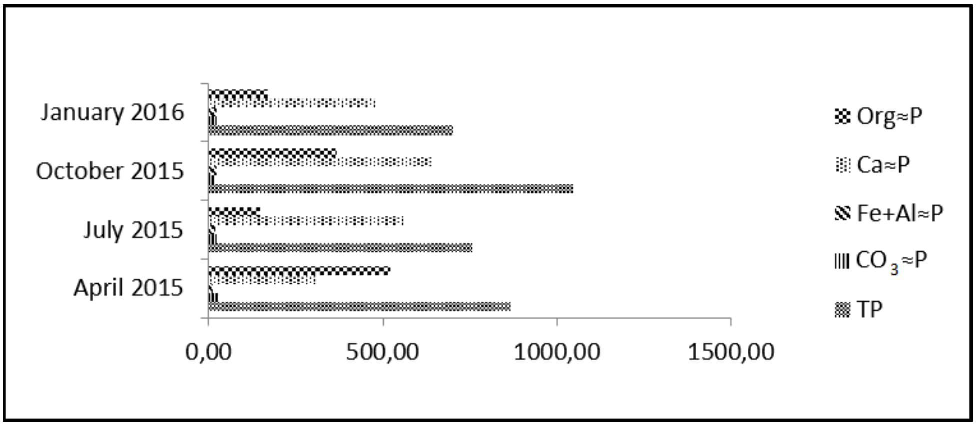

Figure 2. *Seasonal variation of sediment phosphorus fractional composition (µg g^{-1} DW^{1}) in 1st station (a) and in 2nd station (b) of Lake Mogan*

The average values for phosphorus release were calculated every month at each station during the entire research period. The phosphorus release values from the sediment varied between 0.1754-1.1249 mg $m^{-2}day^{-1}$, with the lowest value measured in July at the 2nd station and the highest value seen in January at the 1st station (*Figure 3*).

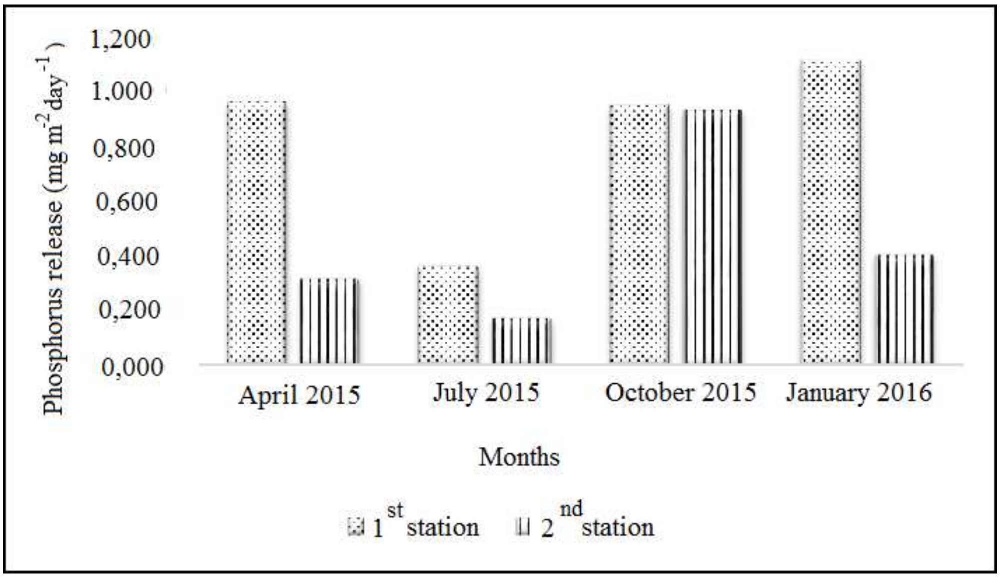

Figure 3. *Monthly and spatially phosphorus release in lake Mogan*

In *Figures 4*, *5* comparison of the present study regarding some sediment chemical composition (total phosphorus, phosphorus fractions, organic material and total iron) with some earlier study data (Topçu and Pulatsü 2008; Pulatsü et al., 2009) considering the common station was presented.

The differences of sediment porewater and sediment overlying water SRP concentrations between the current study and the earlier researches could be followed in *Figure 6* notwithstanding, the comparative phosphorus release estimations of the present and the earlier data were shown in *Figure 7*.

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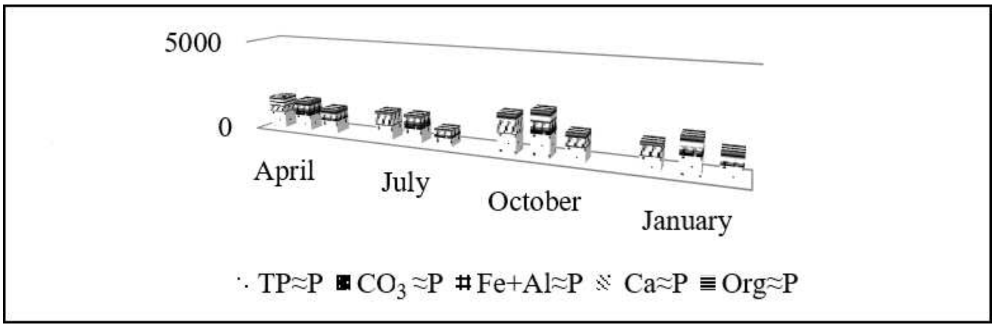

***Figure 4.** Monthly mean concentrations of phosphorus fractional composition (µg g^{-1} DW^{-1}) in Mogan Lake (1st bar: Present study, 2nd bar: Topçu and Pulatsü (2008), 3rd bar: Pulatsü et al. (2009)*

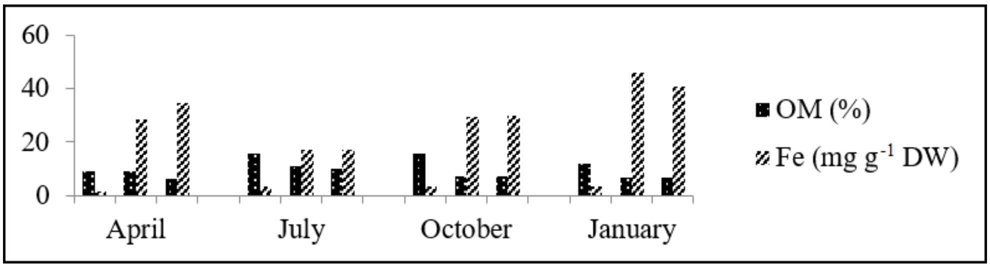

***Figure 5.** Monthly mean concentrations of sediment iron concentration and organic material in Mogan Lake (1st bar group: Present study, 2nd bar group: Topçu and Pulatsü (2008), 3rd bar group: Pulatsü et al. (2009)*

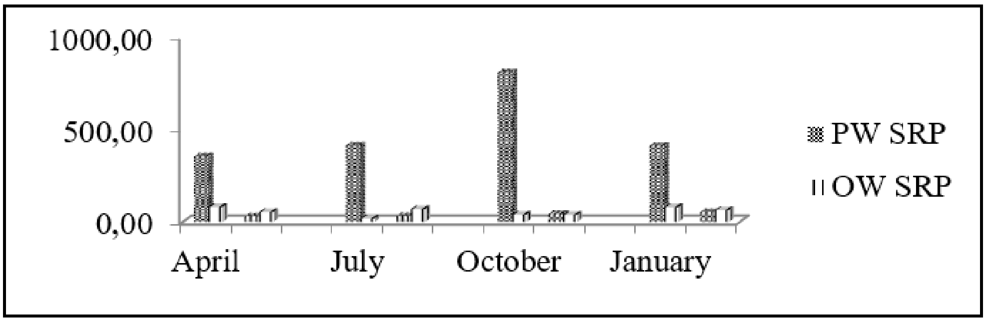

***Figure 6.** Monthly mean concentrations of porewater SRP (mg m^{-3}) and overlying water SRP (mg m^{-3}) in Mogan Lake (1st bar: Present study, 2nd bar: Topçu and Pulatsü (2008)*

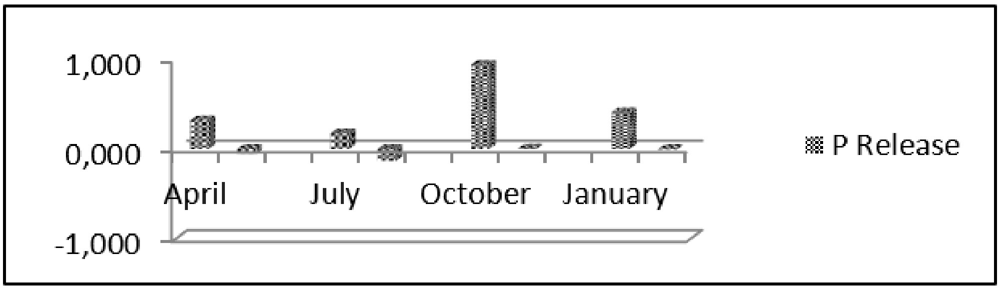

***Figure 7.** Monthly mean concentrations of phosphorus release estimation (mg $m^{-2}day^{-1}$) in Mogan Lake (1st bar: Present study, 2nd bar: Topçu and Pulatsü (2008)*

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Discussion

In a large-scale lake restoration study on European lakes, it was determined that sediments with phosphorus content > 1 mg P g DW^{-1} generally show sediment P release, whereas P retention may be expected for P content < 1 mg P g DW^{-1} (Kelderman et al., 2005). Accordingly, the averge TP concentration in the sediment of Mogan Lake (843 µg gDW^{-1}) is thought to quantitatively demonstrate phosphorus retention by the sediment. The fact that the highest total phosphorus values at both research stations in Mogan Lake were detected in the autumn months supports the findings of Spears et al. (2007) that heavy macrophyte concentration and planktonic detritus are factors leading to high sediment phosphorus levels in the autumn.

Eutrophic Bear Lake (Michigan) is characterized by elevated TP concentrations (Steinman and Ogdahl 2015). There are several possibilities why internal P loading is not more problematic in Bear Lake like this current study; given that the sediments have relatively high TP concentrations. Second reason was the well-mixed water column keeping the lake relatively free of hypoxic conditions. The final reason was the high sediment TFe concentration. Mogan Lake's findings coincide with the above researcher's reasons for the low P internal loading in Bear Lake.

When the findings of Boyd et al. (1994) and Istvanovics (1994) are taken into account, it can be seen that in Mogan Lake's sediment, the variation of the quantity of organic matter between 9 and 16 %, the total carbon concentration (7.11 %) and the limestone sediment composition are important elements contributing to phosphorus retention in the sediment. Phosphorus chemistry in aquatic ecosystems is generally controlled by phosphorus and iron interactions. In this study sediment TFe concentrations range (2-3 mg g DW^{-1}) was lower than both Topçu and Pulatsü (2008) and Pulatsü et al. (2009)'s findings (17-46 mg g DW^{-1}), nevertheless, LOI values (9-16 %) respecting the present study were found to be higher than the previous studies data (6-11 %) in Mogan Lake. When it comes to the levels of iron and organic matter in the sediment of Mogan Lake, it can be said that, in line with Temporetti and Pedrozo's (2000) findings, the sediment's organic matter plays a more important role than its iron concentration in controlling phosphorus release from the sediment.

Moreover, while dissolved oxygen concentrations varied between 6.31-15.72 mg L^{-1} during the study, they never fell to a value low enough to instigate phosphorus release from the sediment into the lake water. Besides this, it can be said that while the pH values found (7.72 - 9.56) were close to the range reported by Lehronta and Heiskanen (2003) (pH>8.5-10), this in itself is not enough to affect phosphorus retention.

Phosphorus fractionation is a good way to understand phosphorus origins and transformation in the sediments in the shallow lakes. Sediment phosphorus fraction forms are potentially mobile and may eventually be released into the lake water (Yuan et al., 2014). The order of phosphorus fractions that are effective in preventing phosphorus release from the sediment into the water in Mogan Lake is Ca≈P>Org≈P>CO_3≈P>Fe+Al≈P except April. Topçu and Pulatsü (2008) reported the sequence of fractional distribution of phosphorus in the sediment of the same lake, regarding the general mean monthly values, as Org≈P>Ca≈P>Fe+Al≈P>CO_3≈P. Among these, it can be told that the fractions with the greatest effect on phosphorus retention in the sediment are calcium-bound and organically-bound phosphorus fractions in general. In this context, considering the common station sediment phosphorus fractional composition showed an irregular distribution.

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Taihu Lake (in the southeast part of the Yangtze River Delta), a large shallow lake, serves as an important source for drinking and irrigation water, moreover a great amount of wastewater has been discharged into the lake with increased urbanization, industrial and agricultural activities in the watershed and the fractional composition of sediment phosphorus was in the descending order: Ca-bound P>Fe bound P>Organic P>Labile P (Yuan et al., 2014). Mogan Lake's sediment important phosphorus fraction coincides with the above researcher's data. It is thought that Mogan Lake's basin of limestone rock and the apatite phosphorus fractions (Ca≈P) originating from this rock promote phosphorus retention by the sediment. However, carbonate-bound phosphorus fractions (CO_3≈P) play an especially important role in clay-rich sediments, and thus represent an important phosphorus fraction in phosphorus retention (Shresta and Lin 1996; Dittrich et al., 2013). The clay-rich composition of the sediment at both of the stations in this study (65 % and 69 %) increased the role of the CO_3≈P fractions in keeping phosphorus release at a low level.

The concentration level of pore water SRP in this study was very high in comparison to Topçu and Pulatsü (2008)'s findings. Moreover, overlying water SRP concentrations of this study was found to be generally higher than the previous study except July. The lowest phosphorus release values in Mogan Lake were measured in July, and the fact that SRP values in the sediment pore water did not show an increase seem to support this finding. SRP values in the sediment pore water in this study reached their maximum values (960.56 mg m^{-3}) in January 2016 due to factors such as death and decay of macrophytes. The increase, especially during the summer, in phosphorus concentrations in the sediment pore water in shallow lakes is a result of phosphorus loading from the sediment (Spears et al., 2007). The fact that SRP concentrations in the overlying water in Mogan Lake were at their highest during the spring and at their lowest values (17.46 mg m^{-3}) during the summer months coincides with the finding that the lowest phosphorus release from the sediment was seen during the summer.

Zamparas and Zacharias (2014) stated that priorly the perpetuity to proceed to a restoration method, like monitoring of external and internal nutrient loads should initially be carried out. In this manner the authors believe that defining the reference conditions of the ecosystem and choosing which method to use in order to have the desired results will be more easier. Although no routine management strategy exists for Mogan Lake, the local government has carried out efforts such as reducing the external pollutant load and diverting running water into the lake to accelerate the flushing of the lake water, and has also occasionally carried out macrophyte harvesting and dredging operations. Dredging alone, one of the management applications in Mogan Lake, without the reduction of external phosphorus loading will result in a system eventually returning to its prior condition agrees with the findings of Smolders et al. (2006) and Sharpley et al. (2013).

Phosphorus fluxes between the water column and the sediment layer were largely driven by the redox chemistry of sediment and dissolved oxygen depletion in bottom waters, which is biologically mediated by heterotrophic oxygen consumption and physically controlled by water column structure (Giles et al., 2016). In Mogan Lake phosphorus release values from the sediment were found to be extremely low, varying between 0.1754 – 1.1249 mg P $m^2 day^{-1}$. The oxygenated overlying water of the Mogan Lake was one of the effective factors leading to a low P flux as the above researchers' expressions. However, these values were higher than the phosphorus release values measured between July 2004 and June 2005 in the same lake by Topçu and Pulatsü

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(2008). The fact that phosphorus release in the lake has increased over the past ten years but still remains at a generally low level together with the above-mentioned management activities in Mogan Lake.

Conclusion

In conclusion, it is clear that the macrophyte harvesting and dredging operations still being undertaken by the local government with the purpose of controlling eutrophication have not had a strong effect on the sediment-sediment overlying water phosphorus mobility. Moreover, considering the last 10 years study, related to the release of phosphorus from the sediment to the water of Mogan Lake, have risen limitedly but does not seem to be extremely high enough to effect the nutrient level of the lake. Consequently, in shallow eutrophic systems such as this one, hotspots of phosphorus accumulation in the sediment are subject to complex interactions with mainly anthropogenic external sources. Nevertheless in order to control the eutrophication process in Lake Mogan, the primary goal should be to determine the adsorption capacity of the sediment in order to estimate the critical internal phosphorus load and take earlier precautions, and the second goal should be to apply a monitoring program on the lake’s trophic level.

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ALGICIDAL EFFECTS OF *ACHILLEA AGERATUM* L. AND *ORIGANUM COMPACTUM* BENTH. PLANT EXTRACTS ON GROWTH OF *MICROCYSTIS AERUGINOSA*

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Abstract. Leaf aqueous (LA) extracts of medicinal plants *Achillea ageratum* L. and *Origanum compactum* Benth. were tested to explore their potential algicidal effects on *Microcystis aeruginosa.* The growth of *M. aeruginosa* and cell morphology changes in response to LA extracts was investigated. The concentrations of total phenols, total flavonoids, and total tannins in LA extracts were analyzed to reveal the potential allelochemical compounds. The results demonstrate that both *A. ageratum* and *O. compactum* LA extracts inhibit *M. aeruginosa* growth in a concentration-dependent way. For both LA extracts, the highest inhibition rate exceeded 80% since the fifth day of exposure. The induction of inhibition effects was translated by a decrease in photosynthetic pigments (chlorophyll-a and carotenoids) and with other morphological modifications. Our results illustrate that both *A. ageratum* and *O. Compactum* LA extracts can offer an effective solution for control of *Microcystis* blooms, and these are recommended in the restoration of aquatic environments contaminated by these types of algal blooms.
Keywords: *Achillea ageratum*; *Origanum compactum*; *algicidal effects; M. aeruginosa; inhibition; algal control*

Introduction

Harmful Cyanobacterial blooms in eutrophic aquatic ecosystems are a frequent phenomenon worldwide. *Microcystis* are the most common bloom-forming cyanobacteria (Mangilia et al., 2010; Douma et al., 2016). These are able to produce hepatotoxins (microcystins) (Wiegand and Pflugmacher, 2005; Douma et al., 2017). Consequently, they are responsible for the intoxication of many organisms and pose adverse effects on water quality and agricultural products destined for animal and human consumption (Mangilia et al., 2010).

The widely used conventional methods to reduce algal biomass are centrifugation, filtration, dissolved air flotation, and flocculation (Liu et al., 2013). These measures involve high costs and may induce secondary pollution. Biological control has emerged as an economic and ecofriendly alternative (Park et al., 2009). Laboratory experiments involving several aquatic plants such as *Myriophyllum spicatum*, *Najas minor* (Zhang et al., 2014), *Stratiotes aloides*, and *Sagittaria trifolia* (Li et al., 2016) have demonstrated inhibitory effects on cyanobacterial biomasses. On the other hand, the algicidal properties of terrestrial plants received less research attention, except for rice hull (Park et al., 2009), barley straw (Ball et al., 2001), mandarin skin and banana peel (Chen et

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al., 2012), and *Ailanthus altissima* tree bark (Meng et al., 2015). In addition, the application of terrestrial medicinal plants for the biological control of invasive or harmful algal blooms remains very limited. However, several species have shown positive bioactivity and demonstrated antioxidant, antibacterial, antifungal, antiviral, and anti-insecticidal potentials (El Bouzidi et al., 2011; Dutra et al., 2016). Yi et al. (2011) have reported the algicidal activities of several Chinese medicinal plant species, including *Salvia miltiorrhiza*, *Acorus tatarinowii*, *Polygonumcuspidatum*, *Phellodendrona murense*, and *Crataegus pinnatifida*. Ye et al. (2014) showed that aqueous extracts of *Phellodendri chinensis* and *Scutellaria baicalensis* have a potential to control *Microcystis* growth.

According to phytochemical characterization, the most important bioactive constituents of medicinal plants are phenolic compounds, fatty acids, and alkaloids (Yang et al., 2015). Among all polyphenols, flavonoids and tannins exhibited an intensive inhibitory effect on *M. aeruginosa* (Chen et al., 2012).

Medicinal flora is very common in the Mediterranean area. Countries such as Morocco have a vast biodiversity of medicinal plants, which have the feasibility to be cultured (El Bouzidi et al., 2011). Among these plants, *A. ageratum* and *O. compactum* are of particular interest due to their pharmacological properties and bioactive products they contain (El Babili et al., 2011; Zhang et al., 2014).

A. ageratum is an *Asteraceae* medicinal shrub. It has demonstrated antibacterial (De la Puerta et al., 1996), antifungal (El Bouzidi et al., 2011), anti-inflammatory, analgesic, and antipyretic activities (Gómez et al., 1999). *O. compactum* is a Lamiaceae herbaceous plant native to the Mediterranean region. Previous pharmacological studies have reported their antioxidant and antimalarial activities (El Babili et al., 2011), and also antimicrobial properties against many bacteria and fungi that cause human infections (Charai et al., 1996).

The aim of this study was to assess the algicidal effects of *A. ageratum* and *O. compactum* on toxic bloom-forming *Microcystis aeruginosa*. The experiments focused on (i) growth inhibition, (ii) morphological and physiological changes, and (iii) characterization of total polyphenols (TPs), total flavonoids (TFs), and total tannins (TTs) as potential allelochemicals. To our knowledge, this is the first report of the algicidal effects of these two medicinal plants.

Materials and methods

Materials

M. aeruginosa was isolated from the eutrophic Lalla Takerkoust reservoir (31°21'36" N; 8°7'48" W), Morocco, in October 2015. Using Z8 medium, *Microcystis* was cultured in laboratory at 26±2°C under light intensity of 65 $\mu Em^{-2}S^{-1}$, with a light/dark cycle of 15h/9h. The aerial parts of *A. ageratum* and *O. compactum*, collected in April 2016 from two locations (Demnat and Ourika in High Atlas of Marrakesh area, respectively), were washed under tap water and then rinsed several times with distilled water to remove debris and epiphytic microbes.

Preparation of LA extracts

LA extracts of *A. ageratum* and *O. Compactum* were prepared according to the methods described by Ball et al. (2001), slightly modified by Li et al. (2016). Briefly,

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10 g was cut into small pieces in 100 ml distilled water under agitation (37°C; 40 min). The solution was filtered through a glass fiber paper (Whatman GF/C, 0.22 µm). Then, the filtrate was adjusted with sterilized distilled water to 100 ml and maintained at 4°C as an aqueous extract.

Determination of TPs, TFs, and TTs in LA extracts

The concentration of TPs was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965). Briefly, 0.5 ml of the extract was added to 0.5 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) in water, and then 0.5 ml sodium carbonate solution (20% w/v) was added. The mixture was left for 1 h at room temperature and absorbance was measured at 760 nm.

The concentration of TFs was determined using the method described by Kim et al. (2013). Briefly, 500 µl of aqueous extract was mixed with 500 µl distilled water. Then 150 µl sodium nitrite solution (5%) was added, followed by 150 µl aluminum chloride solution (10%) after 5 min. Test tubes were incubated for 11 min at ambient temperature, and then 500 µl sodium hydroxide (1 M) was added. The mixture was vortexed and absorbance was determined at 510 nm.

The concentration of TTs was determined using the Folin-Denis test described by Salunkhe et al. (1990). Briefly, 1 ml of the aqueous extract was added to 75 ml distilled water, and then 5 ml Folin Denis reactif (Sigma-Aldrich) solution and 10 ml sodium carbonate solution were added. The mixture was vortexed and absorbance was determined after 30 mn at 760 nm.

Experimental design

Six concentrations (0% control, 0.1%, 0.25%, 0.5%, 0.75%, 1%; V/V) of *A. ageratum and O. compactum* LA extracts were prepared in six groups of Erlenmeyer flasks (500 mL) containing Z8 medium to a final volume (300 ml). Each flask was inoculated by a volume of *M. aeruginosa* in exponential growth phase to make an initial density of 1–2 × 10^6 cell/ml. The cultures were incubated at 26±2°C, illuminated in 15h/9h light-dark cycle with fluorescent tubes (65 $\mu Em^{-2}S^{-1}$) for 8 days. All the experiments were conducted in triplicates.

Biomass estimation

The growth of *Microcystis aeruginosa* under different treatment concentrations was quantified every day using a Malassez hemocytometer. Inhibitory rate (IR) of *M. aeruginosa* growth was determined according to the following equation: IR (%) = (N_0–N/N_0×100, where N_0 and N (cells/ml) are cell density in treatment and control cultures, respectively.

Pigments determination

Chlorophyll-a and carotenoid pigments were extracted with 95% ethanol at 4°C for 48 h and then determined using a UV spectrophotometer (Carré 50 Scan) at 470, 649, 665 nm. Pigments were calculated according to the Lichtentaler and Wellburn method (1983). The following formulas were used to calculate their concentrations:

$$\text{Chlorophyll-a} = 13.95\times OD665 - 6.88\times OD649;$$

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Carotenoids = [(1000×OD470) – (2.05×chlorophyll-a)] / 2

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) using SPSS v20.0 (Windows 2010). One-way ANOVA and Tukey's test were used to test differences between exposure concentrations and control at p = 0.05. Correlation coefficients were calculated between cellular density and concentrations of PTs, FTs, and TTs.

Results

Effects on growth of M. aeruginosa

The inhibitory effects of both *A. ageratum* and *O. compactum* LA extracts, at different concentrations, on *M. aeruginosa* cultures are shown in *Figure 1A* and *B*. In contrast to the control group, the cell biomass of *M. aeruginosa* in all five tested concentrations (0.1%, 0.25%, 0.5%, 0.75%, and 1%; V/V) was significantly reduced during the 8-day test period (p<0.05). The reduction was more remarkable for the three highest concentrations, especially during the last days (*Fig. 1A-B*). Under *A. ageratum* and *O. compactum* LA extracts, *Microcystis* growth appeared to have strongly reduced at 0.5–1% and 1% concentrations, respectively. At these concentrations, the inhibitory rates exceeded 80% since the fifth day and reached more than 90% during the last day (*Table 1*).

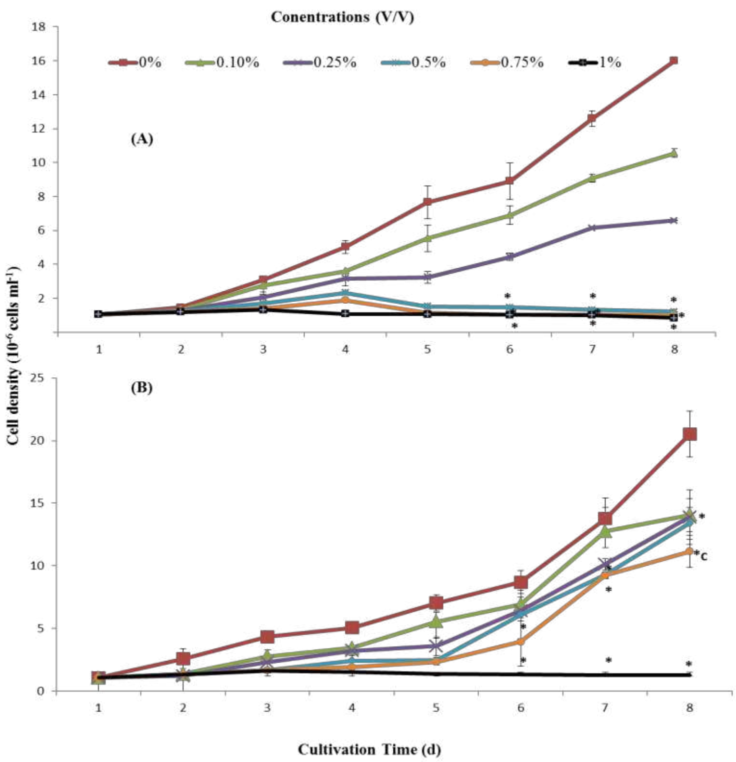

Figure 1. *Effects of different concentrations of A. ageratum (A) and O. compactum (B) LA extracts on Microcystis aeruginosa growth. Errors bars represent standard deviation (n=3). *p<0.05 indicates significant difference compared to controls.*

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Table 1. *Inhibitory effects of A. ageratum and O. Compactum aqueous extracts on M. aeruginosa growth.*

	PT [1]	FT [2]	TT [3]
plant	*A. ageratum*		
Concentrations	79,19 ± 8,37	28,17 ± 5,5	0,08± 0 ,005
Coefficient of correlation	-0,044	**0,518**	-0,803
plant	*O. compactum*		
Concentrations	69,03±5,99	23,64±13,69	0,03±0,005
Coefficient of correlation	**0,994**	-0,285	**0,86**

([1]): µg gallic acid equivalent ml^{-1}aqeous extract, ([2]): µg catechin equivalent/ml aqueous extract, ([3]): µg tannic acid equivalents ml^{-1}aqeous extract

Morphological changes in M. aeruginosa

The morphological changes in *M. aeruginosa* cultures under treatments were photographed and presented in *Fig. 2*. In the control group, *M. aeruginosa* colonies were clearly structured with regular surfaces. Their cell forms were rounded, clearly vacuoled, and pigmented, with cell diameter 2.8–3.5 µm by the end of treatment period. However, under high concentrations (0.75% and 1%), *M. aeruginosa* colonies lost their regular form, becoming floating aggregates (2.5–3.4 µm cell diameter) during the first days of culture. Then, for a heterogeneous culture with planktonic cells (1.1–1.5 µm) and sediment mass with anaform cells which the observed cells were destroyed and shrinking cell surface, especially, in the end of the test period (*Fig. 2*).

Effects on pigments as physiological indicators

For both *A. ageratum* and *O. compactum*, two photosynthetic pigments were measured (chlorophyll-a and carotenoids) that would act as physiological indicators. For the control, the chlorophyll-a and carotenoids concentrations were dose-dependent (*Fig. 3*). These decreased negatively with the augmentation of extract concentrations. For *O. compactum*, a significant difference in concentrations between the treatment and control groups was observed ($p<0.05$) (*Fig. 3A2–B2*). The pigments appeared to be strongly inhibited at 1% concentration since the sixth day. For *A. ageratum*, the concentrations 0.5%, 0.75%, and 1% seemed to be clearly inhibitory since the fifth day (*Fig. 3A1–B1*).

Phytochemical characterization

A. ageratum demonstrated higher values of TPs, TFs, and TTs compared to *O. compactum*. In addition, a significant correlation between the IRs of three high

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concentrations (0%, 5%, 1%) and TFs concentrations (coef>0,5) for *A. ageratum* , and between those of TPs and TTs concentrations (coef> 0,8) for *O. compactum* (*Table 2*).

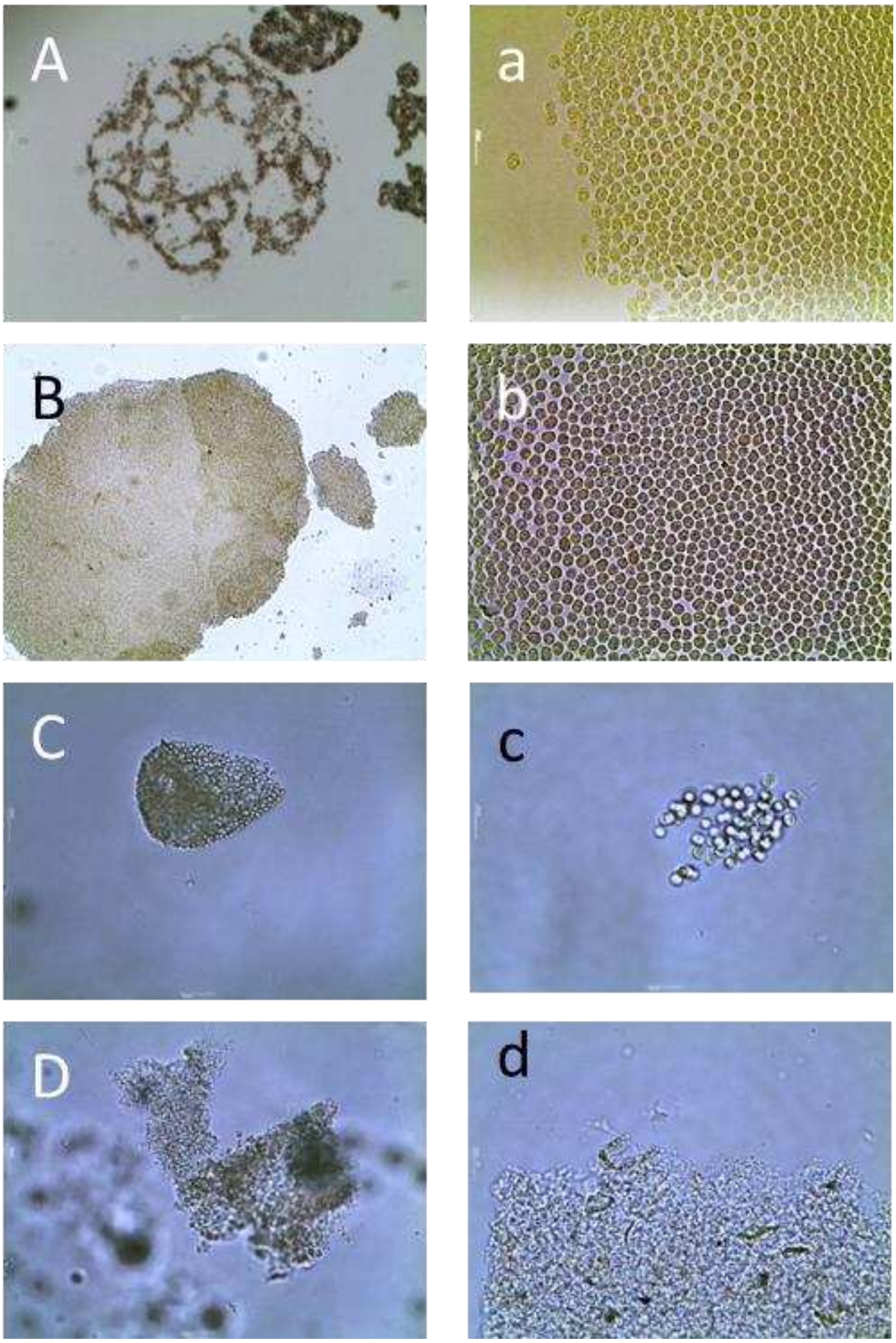

Figure 2. *Modification in M. aeruginosa colonies (A–D)(Gr. ×40) and cells (a–d)(Gr. ×1000) under treatments. (A) M. aeruginosa in normal growth (control), (B) formation of flotants and aggregate colonies, (C) colonies less vacuolated and breaking down, (D) sediment cells, completely devacuolated and decomposed.*

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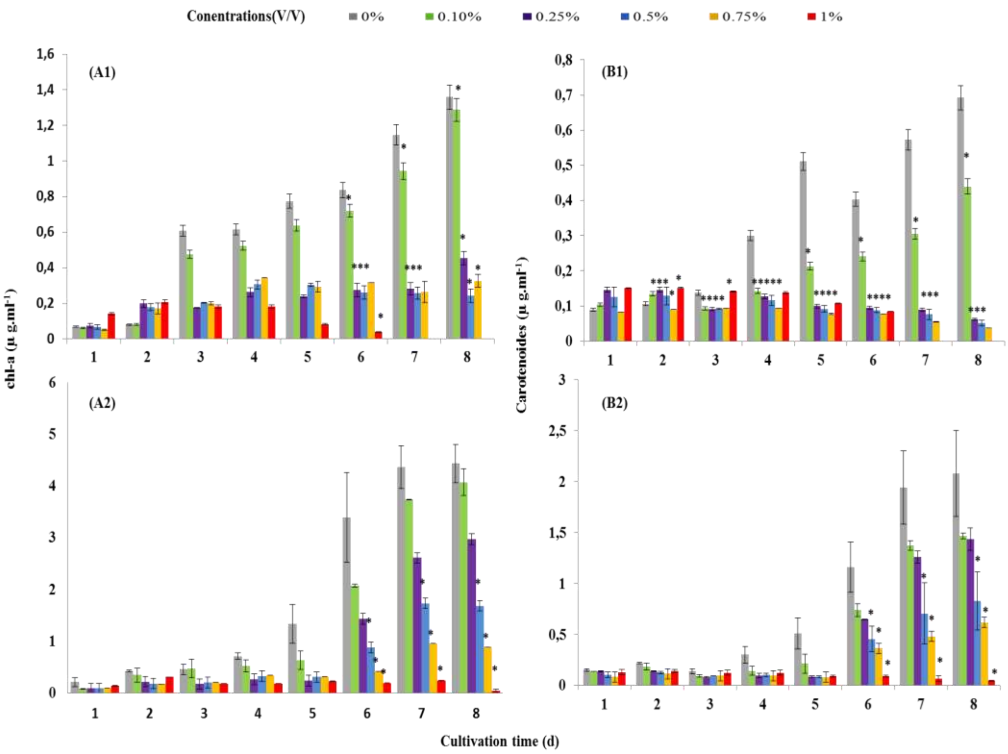

Figure 3. *Effects of A. ageratum (A1, B1) and O. compactum (A2, B2) LA extracts on chlorophyll-a and carotenoids in M. aeruginosa cultures. Data are means + SD (n=3). *p <0.05 indicates significant differences compared to controls.*

Table 2. *TP, TF, and TT amounts in different A. ageratum and O. compactum LA extracts; and correlations between all amounts and IRs of three high concentrations (0.5%, 0.75 %, 1%) after 8 days of exposure.*

	Cultivation Time (d)	1	2	3	4	5	6	7	8
	Concentrations (%)	Inhibitory rate (%)							
A. ageratum	0.1	-1	9	11	28	28	23	28	34
	0.25	-1	12	34	37	58	50	51	59
	0.5	1	15	46	**54**	80	84	89	92
	0.75	0	16	**55**	62	85	88	92	94
	1	-1	19	**57**	79	86	88	92	95
O. compactum	0.1	-1	48	36	32	21	20	7	31
	0.25	-1	53	47	36	49	26	27	32
	0.5	1	48	62	52	65	30	33	35
	0.75	0	48	62	62	67	55	33	46
	1	-1	49	62	70	80	85	91	94

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Discussion

Both *A. ageratum* and *O. compactum* LA extracts acted negatively on *M. aeruginosa* growth (*Fig. 1*). This inhibitory effect appeared to be dose-dependent.

A. ageratum showed high IRs with three high concentrations (0.5–1%) compared to *O. compactum* (only 1%). For these last concentrations, since the fifth day, a marked IRs were observed for *A. ageratum and O. compactum*, exceeding 90% at the end of the experiment for each plant. This finding can be used for the optimization of extracts for a recommended treatment, not envisaging a complete reduction of *M. aeruginosa* biomass.

The maximum IRs of high concentrations remain similar to those observed in previous works. *Sagittaria trifolia* aqueous extract showed inhibition on *M. aeruginosa* growth with IRs exceeding 70% after 6 days (Li et al., 2016). Ye et al. (2014) studied the effects of several Chinese herbal aqueous extracts on *M. aeruginosa* and found that the maximum IRs were 51–98% after 10 days. Thus, the inhibitory effect of our aqueous extracts is similar to that reported in previous works (Chen et al., 2012; Zhang et al., 2014; Meng et al., 2015; Li et al., 2016).

The inhibition of photosynthesis is a prominent mode of action for many allelopathically active compounds (Gross, 2009). The effects of plant extract on chlorophyll-a and carotenoids are shown in *Figure 3*. Some studies have shown the negative effects of the extracts on chlorophyll-a (Meng et al., 2015). The disturbance in photosynthesis thus affects the growth and reproduction of *M. aeruginosa* (Li et al., 2016).

The phytochemical characterization of the aqueous extracts shows important amounts of TPs, TFs, and TTs (*Table 2*). FTs, and PTs and TTs contribute to the inhibitory action of *A. ageratum* and *O. compactum,* respectively. These results agree with previous works showing the effects of PTs, FTs, and TTs on *M. aeruginosa* (Yan et al., 2011; Chen et al., 2012). Furthermore, other compounds have been identified during the characterization of *O. compactum* (carvacol and thymol; El Babili et al., 2011), and *A. ageratum* (artemisyl acetate; El Bouzidi et al., 2011), which contribute to the algicidal process.

As concluded by previous studies, the allelochemical products inhibit cell growth by attacking the reactive oxygen species on cell membranes, degrading the unsaturated phospholipids, and consequently increasing their permeability, leading to a disruption of cell organization (Meng et al., 2015). Under optimum stress, the perturbations of the antioxidant defense system lead to the inhibition of photosynthesis and oxygen evolution through interactions with the components of PS II (Einhellig, 1995) and finally slowing down cell death.

In our study, growth inhibition was well demonstrated by morphological changes in treatment groups (*Fig. 2*). In addition, the cells in treatment groups decreased in size and lost a significant volume of vacuoles (*Fig. 2*). Some have completely destroyed and sedimented; others resisted by attempting to escape through a floating agglomeration. The aggregation trend of cells in high concentrations of extracts appeared to be a response to stress conditions. The growth inhibition accompanied by photosynthetic pigments and morphological changes in *M. aeruginosa* are indicators of physiological alterations under a stress environment (Yan et al., 2014; Meng et al., 2015; Li et al., 2016).

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Conclusions

The present work demonstrates the inhibitory effect of LA extracts of *A. ageratum and O. compactum* on *M. aeruginosa* growth. In addition to their antimicrobial, anti-inflammatory, analgesic, and antipyretic potentialities; and according to our results, these two medicinal plants can be recommended as an ecofreindly solution to restore the aquatic environments contaminated by *M. aeruginosa* blooms. Other future approaches will be necessary to specify and optimize the allelochemical agents, as well as the conditions of interactions with other potential components, especially others pathogens microbes, in the aquatic ecosystem.

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Conflict of Interest. The authors declare no conflict of interest.

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ASSESSMENT OF DNA DAMAGE USING RANDOM AMPLIFIED POLYMORPHIC DNA IN VEGETATIVE-STAGE BEAN (*PHASEOLUS VULGARIS* L.) GROWN UNDER A LOW FREQUENCY ELECTROMAGNETIC FIELD

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Abstract. Electromagnetic field produced by a moving electrically charged object, can be measured everywhere in the environment and it acts as an important effective stress factor on growth and development of plants. Plants are organisms exposed to different levels of biotic and abiotic environmental impacts and are able to distinguish and respond to their surrounding environment with a high specificity. This study aimed to determine the genotoxic effect of a low frequency electromagnetic field (EMF) on the genomic material of plants. Two groups of bean seeds (wet and dry) were subjected to an electromagnetic field of 4 mT. RAPD profiles of EMF-treated seeds showed differences in banding patterns compared to those of the control. There is no significant different between dry and wet treatments. This result forcefully indicated that genomic template stability and band sharing index was significantly affected by the EMF-induced stress in treated seeds. It was concluded that DNA polymorphism detected by RAPD fingerprinting thus could be used as a potential molecular marker for the assessment of electromagnetic field-induced genotoxicity effects in plants and different treatments including chemical and physical motivators.

Keywords: *EMF exposure, genomic template stability, genotoxicity, RAPD, Fabaceae*

Abbreviations: *BSI – band sharing index; GTS – genomic template stability*

Introduction

In recent decades, physical methods based on the usage of electromagnetic fields are developed in the industry, agriculture, etc., and finally their waves distributed in the environment (Racuciu and Creangia, 2005). Levels of electromagnetic fields (EMF) from artificial man-made sources have increased progressively in the past decades and human societies are not aware that magnetic fields and plant development are related to each other. This relationship can be used in agriculture by treating the seeds, water, soil and nutrients in the soil (Rio and Rio, 2013; Cucurachi et al., 2013).

There is a rich literature describing the biological influence of low frequency electromagnetic fields upon vegetal organisms. But the responses elicited in various plant species appear to depend in a complex way on both the physical parameters of the irradiation source (frequency, power density, pulses or continuous waves, duration of exposure, etc.) and the conditions of the biological materials (vegetation stage, pretreatment, environment, etc.), nevertheless different biological parameters may lead to the revealing of different aspects of the interaction between the electromagnetic waves and the irradiated material (Ursache et al., 2009).

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Electromagnetic fields induce several cellular stress responses and change different cellular components such as membranes, proteins, enzymes and genome (Aksoy et al., 2010). Higher plants have been used as efficient bio-indicators of genetic toxicity of environmental pollutants. Several researches have used the chromosome aberration, micronucleus, or comet assays to estimate the effects of genotoxicity on plants (Steinkellner et al., 1999; Angelis et al., 2000; Yildizet al., 2009). Recently, advances in molecular biology have led to the development of a number of selective and sensitive assays for genomic material analysis in eco- genotoxicology. DNA based techniques (RFLP, QTL, RAPD, AFLP, SSR and VNTR) are used to examine the variation at the DNA sequence level (Liu et al., 2007; Enan, 2007). Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR)-based technique that gives clearance for swift and efficient comparisons of genomic DNA. This technique is used extensively for species classification, genetic mapping, phylogeny and also it has been successfully used to study genotoxicity (e.g. DNA change, point mutation, insertions or deletions and ploidy variation) in different plants (Kumari et al., 2009; Liu et al., 2009; Cenkci et al., 2009).

The common bean [*Phaseolus vulgaris* L. (cv. D81083)], was used as plant material in this study that it is the major source of protein and minerals for example iron, magnesium and etc. in the world. Bean is a diploid (2n = 22), in fact it is important genetic model that has been widely used in physiological, biochemical and molecular analyses of toxicology (Enan, 2007).

The aim of the present study was to evaluate the DNA changes induced by low frequency electromagnetic field (4 mT) using RAPD technique in the vegetative stage.

Material and Method

Phaseolus vulgaris L. (cv. D81083) seeds were prepared from khomain research center, Arak, Iran. The seeds were surface- sterilized using sodium hypochlorite at 5% for 10 min, washed two times with distilled water and dried at room temperature. The seeds were divided into two groups, one group was immersed in distilled water for 12 h (wet seeds) while the second group remained dry (dry seeds). Each group was further divided into three subgroups: two sub groups were exposed to EMF (once treated and twice treated subgroups), while the third, non-exposed seeds were used as control (not treated subgroup).The seeds of once treated sub group were exposed to a field of 4mT intensity for 45 min, while those of twice treated subgroup were exposed twice to a 4mT field with a two-hour interval, each time for 45 min. Three replicates were used in the experiment with 30 seeds in each treatment. The electromagnetic field was performed by a locally designed generator which its electrical power was provided by a 220 V and 0.1 A, AC power supply. This system consists of a PVC cylinder with 20 × 20 cm (diameter and length) and 300 turn coil of copper wire. The samples were placed in the middle of a horizontally fixed coil and for exposure. The temperature was measured with a thermometer to be 22±1°C.Then treated and untreated seeds were grown in petri dishes in a growth room with temperature controlled at 28±1° C and a 16 h light/8h dark cycle.

The leaves of plants in vegetative stage were used for genomic DNA extraction. Silica gel was used for the dehydration of fresh leaves and were stored at -20°C. DNA was extracted following the cetyltri methyl ammonium bromide (CTAB) method as described by Saghai-Maroof (Saghai-Maroof et al., 1984). Briefly, fresh plant sample was ground to a fine powder in liquid nitrogen, mixed with 1 mL of CTAB extraction

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buffer [2% hexa decyl trimethyl- ammonium bromide, 100 m M Tris–HCl, (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 1% PVP-40 and 0.4% 2-mercaptoethanol]. Nineteen 10-mer oligo nucleotide primers (macrogen, Tehran) were used for characterization of genotypes (*Table 1*). *In vitro* amplification using polymerase chain reaction (PCR) was performed using 50 ng of genomic DNA of each genotype, 1.7 µM primer, 200 µM dNTPs (50 µM of each), 10× reaction buffer (100 mM Tris–HCl, pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, 0.8% Nonidet P40) and 1 U Taq DNA polymerasein a final volume of 20 µl per reaction.

Amplifications were performed in a DNA thermocycler (Uvigene, Uvitech Ltd., UK) programmed for 4 min at 94 °C (initial denaturing step), 40 consecutive cycles each consisting of 45 s at 94 °C (denaturing), 45 s at 36 °C (annealing), 60 s at 72 °C (extension), followed by 1 cycle for 8 min at 72 °C (final extension step).The PCR products were analyzed by electrophoresis on 1.5% agarose gels in 0.5× TBE (90 mMTris base, 90 mM boric acid and 2 mM EDTA) buffer. The gel was run at 10 V, visualized under UV light and photographed using ultracam digital imaging.

Table 1. *Sequences of primers used in this study*

Primer name	Primer sequence	Primer name	Primer sequence
OPA-10	5′-GTGATCGCAG-3′	OPA-17	5′-CACCGCTTGT-3′
OPE-11	5′-CAATCGCCGT-3′	OPB-10	5′-CTGCTGGGAC-3′
OPE-01	5′-CCCAAGGTCC-3′	OPD-07	5′-TTGGCACGGG-3′
OPE-12	5′-TTATCGCCCC-3′	OPG-14	5′-GGATGAGACC-3′
OPA-05	5′-AGGGGTCCTG-3′	OPC-08	5′-TGGACCGGTG-3′
OPA-04	5′-AATCGGGCTG-3′	OPB-07	5′-GGTGACGCAG-3′
OPA-01	5′-CAGGCCCTTC-3′	OPC-02	5′-GTGAGGCGTC-3′
OPG-06	5′-GTGCCTAACC-3′	OPA-09	5′-GGGTAACGCC-3′
OPA-07	5′-GAAACGGGTG-3′	OPA-03	5′-AGTCAGCCAC-3′
OPE-06	5′-AAGACCCCTC-3′		

All amplifications were repeated twice in order to confirm the reproducible amplification of scored fragments and only reproducible bands obtained in repeated experiments were taken into account. The marked changes observed in RAPD profiles (disappearance or appearance of bands, increase or decrease in band intensity in comparison with untreated control treatments) were evaluated (*Table 2*).
Genomic template stability (GTS, %) was calculated as follows:

$$GTS\ (\%) = (1 - \alpha/\mathrm{n}) \times 100 \quad \text{(Eq. 1)}$$

Where α is the average number of polymorphic bands detected in each treated sample and n is the number of total bands in the control. (Savva, 2000)
In order to evaluate the band sharing index (BSI) that is considered as a measure of similarity between two samples, following equation was used:

$$BSI = 2s/(\mathrm{a+b}) \quad \text{(Eq. 2)}$$

where *s* is the number of bands shared between two samples, *a* is the number of bands in the first sample and *b*is the number of bands in the second sample. A BSI value of 1

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indicates that the two samples are identical, while a BSI value of zero indicates that the two samples are totally different (Savva, 2000).

Results

In order to verify the geneotoxic effect of low frequency EMF, the RAPD analysis was performed on DNA extracted from the 30-d-old plants. In total, nineteen 10-mer oligo nucleotide primers of 60-70% GC content were utilized for screening the bean genome for modification in response to EMF. Seventeen primers generated specific and stable results. The total number of bands was 174, 175, 171, 179, 177, 172 in untreated dry seed, once treated dry seed, twice treated dry seed, untreated wet seed, once treated wet seed and twice treated wet seed, respectively, ranged from 100 to 1500 bp. The maximum and minimum of bands observed in untreated wet seed and twice treated dry seed RAPD profiles respectively. On the other hand, seeds in wet condition had more bands than those in dry condition. As observed in *Table 2*, RAPD profiles of the controls have more bands in comparison to treatments.

Table 2.*Changes in total bands in control, and of polymorphic bands and varied bands in EMF-treated seedlings. a: appearance of new bands, b: disappearance of normal bands, c: decrease in band intensity, and d: increase in band intensity. a+b denotes polymorphic bands, and a+b+c+ d denotes varied bands*

No. of primers treatments																		
	Dry seed									**Wet seed**								
	No EMF	**Once EMF**				**Twice EMF**				**No EMF**	**Once EMF**				**Twice EMF**			
		a	b	c	d	a	b	c	d		a	b	c	d	a	b	c	d
OPA-10	6	0	0	1	0	1	1	1	1	6	0	0	0	0	1	1	1	1
OPE-11	8	0	0	0	0	2	3	1	1	8	2	0	0	1	3	4	0	0
OPE-01	8	0	0	0	0	1	2	1	0	8	0	0	0	0	1	2	0	0
OPE-12	12	0	0	0	0	0	2	0	0	12	0	0	0	0	0	2	0	1
OPA-05	10	0	0	0	0	2	1	0	0	10	1	0	0	0	1	1	0	0
OPA-04	13	0	0	0	0	0	3	0	0	12	0	0	0	0	0	1	0	0
OPA-01	13	0	0	0	0	4	3	0	0	13	0	0	0	0	3	3	0	0
OPG-06	10	1	0	0	0	2	1	0	0	10	0	0	0	0	2	1	1	1
OPA-07	9	2	2	0	0	1	3	0	0	11	0	0	0	0	0	3	0	0
OPE-06	11	0	0	0	0	4	1	0	2	11	0	0	0	0	3	2	0	2
OPA-17	8	1	2	0	0	1	1	0	0	8	0	0	0	0	2	0	0	0
OPB-10	5	0	0	0	0	0	2	0	0	7	0	2	1	0	0	5	2	0
OPD-07	8	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0
OPG-14	12	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0
OPC-08	7	0	0	0	0	0	1	0	1	7	0	0	0	0	0	1	0	1
OPB-07	12	2	0	0	0	1	0	0	0	12	0	0	0	0	2	1	0	0
OPC-02	8	0	1	0	0	1	1	0	0	8	0	0	0	0	1	1	0	0
OPA-09	12	1	1	2	0	1	2	2	0	13	0	1	0	0	1	3	0	0
OPA-03	2	0	0	0	0	3	0	0	0	3	0	2	0	0	4	1	0	0
Total bands	174	7	6	3	0	24	27	5	5	179	3	5	1	1	24	31	4	6
a + b		13				51					8				55			
a + b + c + d		16				61					10				65			

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RAPD profiles showed remarkable differences between untreated control and treated plants with apparent changes (disappearance and/or appearance, increase and decrease of band intensity) in the number and size of amplified DNA bands for different primers. The changes in RAPD profiles were summarized for treated seedlings in comparison to their controls (*Table 2*). RAPD pattern screated by OPD-07, OPG- 14 and OPC-08 primers were identical for all treatments and the controls (*Fig. 1*). For OPD-07, OPG-14 primers, there was no change in RAPD profiles in all samples.

RAPD pattern in plants exposed to EMF showed variations in the appearance and disappearance of bands, as well as changes in band intensity in both dry and wet conditions. The number of polymorphic bands (appearance of new bands+ disappearance of normal bands) and varied bands (appearance of new band + disappearance of normal band + decrease in band intensity + increasein band intensity) were 13, 16 and 51, 61 bands in the once EMF treatment and twice EMF treatment in dry condition, respectively. Similarly in the once EMF and twice EMF treatments in wet condition the number of polymorphic bands and varied bands were 8, 10 and 55, 65 bands, respectively.

In the once EMF treatments, the number of new bands was low (7 and 3 new bands in dry and wet, once EMF treatments, respectively), while the number of new bands was high (24 and 24 bands in dry and wet twice EMF treated, respectively) in the twice EMF treatments. The number of disappeared RAPD bands was more at twice EMF treatments (27 and 31 bands) than once EMF treatments (6 and 5 bands). No significant difference was observed between once EMF treatments and their controls. Furthermore, the decrease and increase in band intensity were observed for plants exposed to all EMF treatments (once and twice) used in this experiment for nine of the primers. Changes in band intensity were greater in twice EMF than in once EMF treatments (*Table 2*).

For each treatment group, the value of Band Sharing Index (BSI) and Genomic Template Stability (GTS) compared to their controls were calculated (*Table 3*). Results showed that with increase the time of exposed to the EMF, BSI and GTS values decreased in all treatments that showed the existence of variation among of RAPD profiles in all treatments compared to their controls. The estimated GTS and BSI characters were found to be lower (<1.00) in all treatments compared to the controls. Similar results were detected in twice EMF treatments compared to once EMF treatments. According to these characters, the seeds treated with once EMF showed the nearest distance (BSI: 0.92, 0.96. GTS: 92.52%, 95.53%) to their controls, but twice EMF treatments showed the furthest distance (BSI: 0.71, 0.70. GTS: 70.68%, 69.27%).

Table 3. *Effect of EMF on Band Sharing Index (BSI) and Genomic Template Stability (GTS) of treated bean seeds compared to the control*

	Treatments			
	Dry seed		**Wet seed**	
Character	**Once EMF**	**Twice EMF**	**Once EMF**	**Twice EMF**
BSI	0.92	0.71	0.96	0.70
GST	92.52 %	70.68 %	95.53 %	69.27 %

As indicated in *Table 3*, quantity of GTS and BSI characters are similar. As seen in our general results, wet seeds exposed to an EMF were much more affected than the dry seeds.

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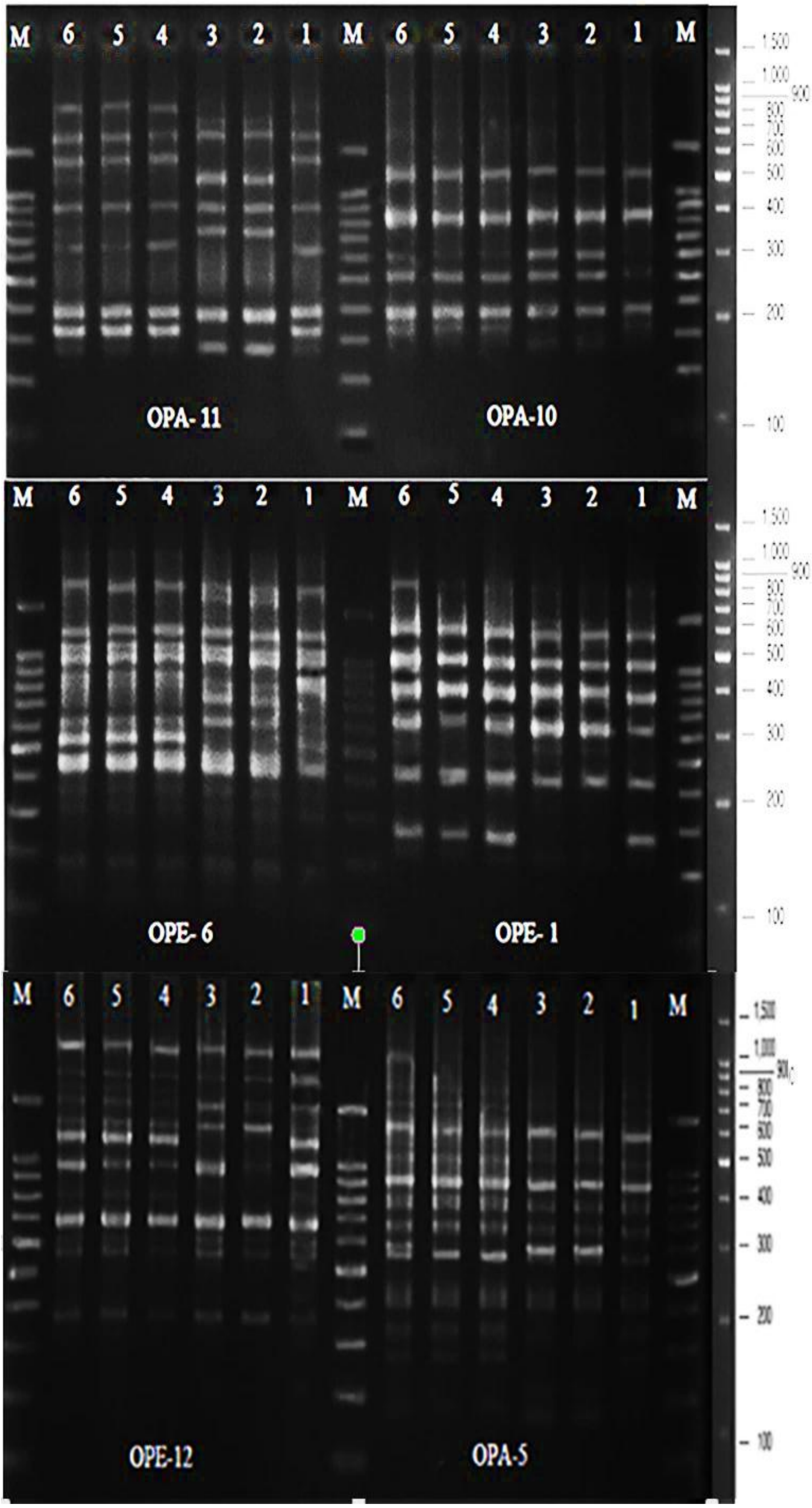

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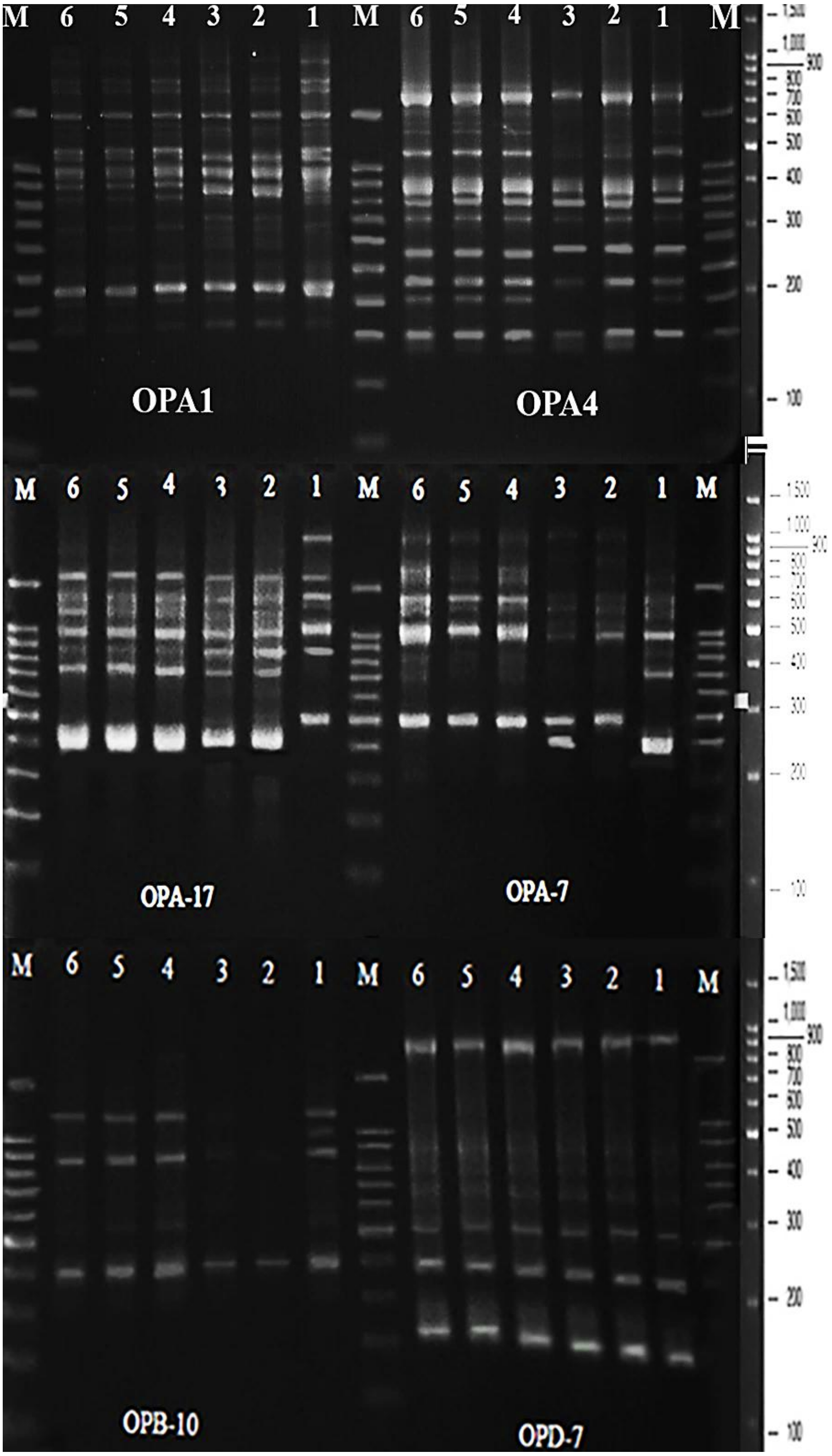

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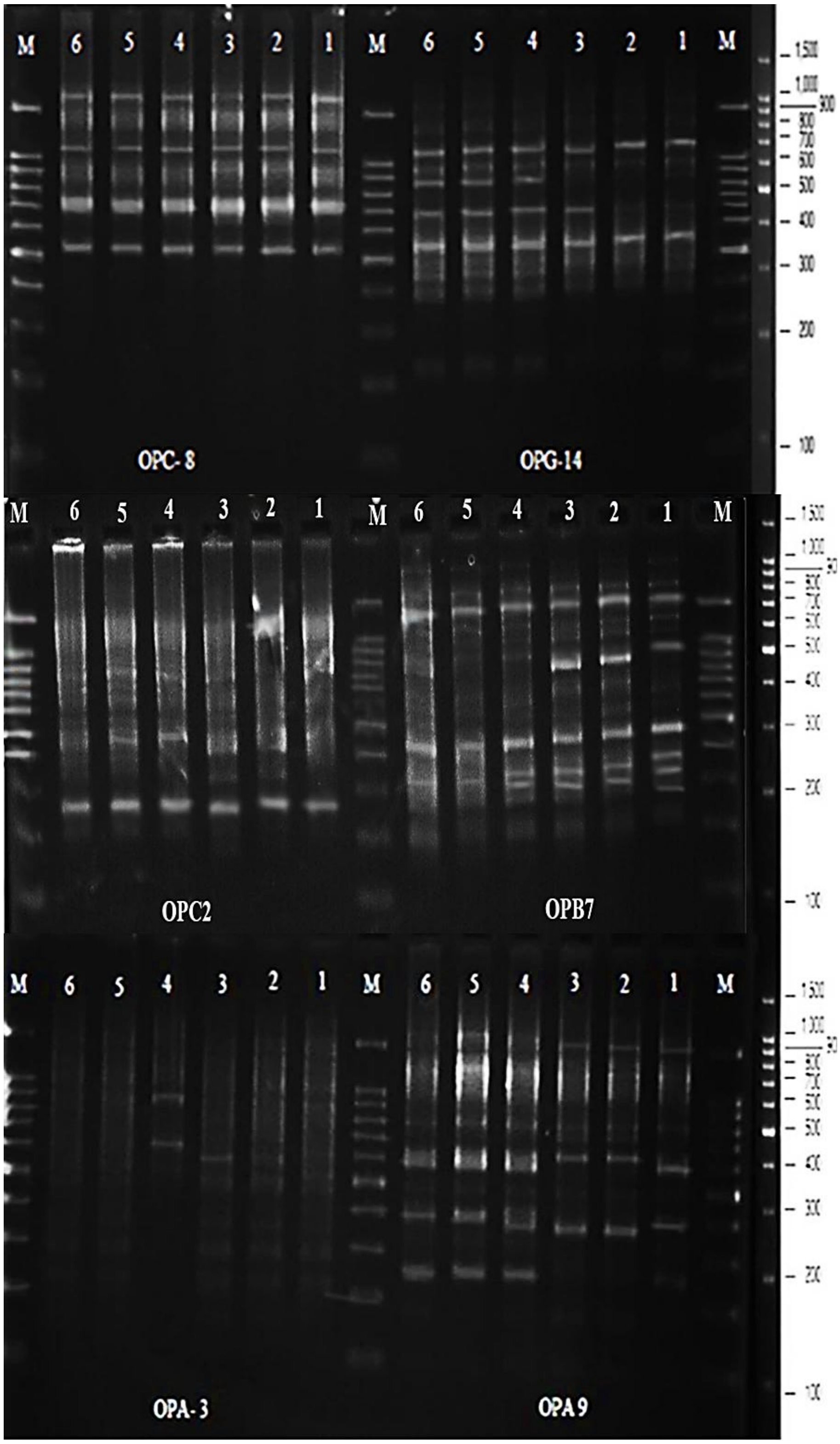

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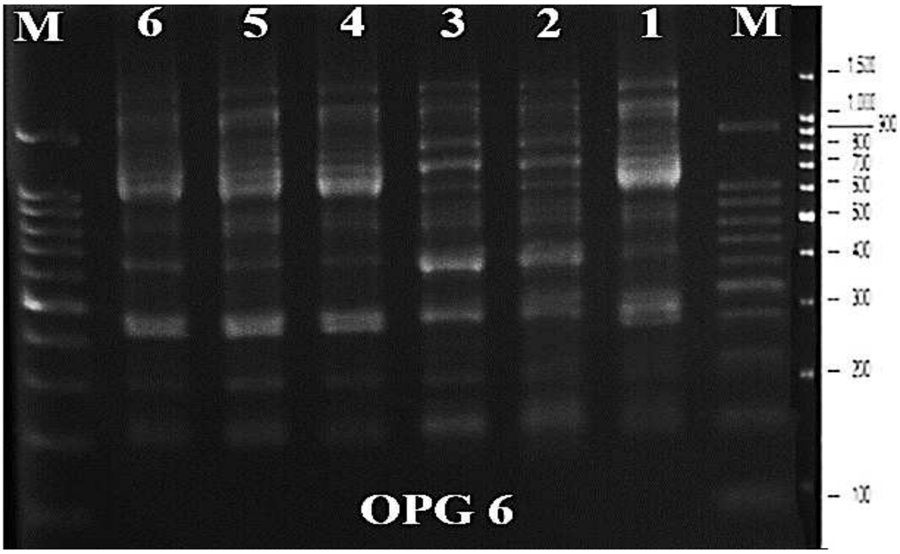

Figure 1. *RAPD profiles in plants exposed to different EMF treatments; the numbers represented the groups. 1: once EMF in dry seed, 2: Twice EMF in wet seed, 3: Twice magnetic field in dry seed, 4: control in wet seed, 5: control in wet seed, 6: once EMF in wet seed, M: DNA ladder (100–1500 bp)*

Discussion

In this study, we focused on the potential of RAPD assay to evaluate genotoxic effects in plants exposed to low frequency EMF for two different durations (a EMFof4mT intensity for 45 min, and a 4mT EMF with two-hour interval, each time for 45 min) and two conditions (dry and wet).

To detect different types of DNA change and genotoxic effect induced by chemical motivators in plants, RAPD method has been utilized extensively (Aksoy et al., 2010; Liu et al., 2007; Enan, 2007). The changes in DNA quality and quantity were detected by RAPD patterns created by randomly primed PCR reactions. These modifications may include changes in oligo nucleotide priming sites and variations in the activity of the *Taq* DNA polymerase. In this study, according to the RAPD profile, the obvious disappearance and appearance of bands produced by EMF in comparison to the untreated controls were considered to detect distinct changes between treatments. Our results showed a greater number of disappearance bands (69 bands) than appearance bands (58 bands) suggesting that the disappearance of normal bands and band intensity may be related to DNA change, point mutations and/or complex chromosomal rearrangements (Wolf et al., 2004). Also the appearance of new bands may unfold a modification in some oligo nucleotide priming sites due to mutations, large deletions, and/or homologous recombinations (Atienzar et al., 1999). The presence of new bands in this experiment may also be the result of genomic template instability related to the level of DNA change, the efficiency of DNA repair and replication (Atienzar et al., 1999).Therefore appearance of new bands could be related to mutations while the lost bands could be due to DNA change (Atienzar and Jha, 2006).

The RAPD patterns acquired with 19 primers were different for controls. Treatment of bean seeds with EMF induced genomics changes in plants. The number of disappeared and appeared bands in the twice EMF was higher than that of the once EMF treatments. Our results (*Figure 1* and *Table 2*) indicated that polymorphism

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increased with increasing EMF exposure time. On the other hand, the genomic template stability and Band Sharing Index in the treatments decreased with increasing EMF exposure time. Therefore, this study revealed the existence of a time exposing- effect relationship. Based on this, a correlation between EMF exposure period and level in EMF-induced DNA change in plants was shown by using the comet assay. Previous studies have also shown that changes in RAPD profiles can be regarded as changes in genomic DNA template stability, and this genotoxic effect can be directly compared with alterations in other parameters (Atienzar et al., 2000). Genotoxicity studies can be elicited qualitatively and quantitatively via the comparison of the control and treated groups. The diagnostic analysis contains band intensity differences or disappearance and/or appearance of RAPD bands are important parameters in RAPD method (Wolf et al., 2004).Although great progress has been achieved in the elucidation of molecular mechanisms of EMF, how plants sense and transduce the signal of EMF stress is still an important to picto be addressed.

Conclusion

In conclusion, our data support the view that RAPD analysis is a highly sensitive method for the detection of DNA change induced by environmental pollutants such as the magnetic fields. Also our results confirmed that low frequency electromagnetic fields have genotoxic effects on DNA structure. Moreover, as the purpose in this research was to establish the existence of DNA change for hazard identification in risk evaluation of genotoxicity studies, the presence in the DNA of any of these varied RAPD profiles can provide adequate evidence for identification of the genotoxic effect.

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CLIMATE CHANGE IMPACTS AND WATER RESOURCES MANAGEMENT: CHEHEL-CHAI BASIN, IRAN

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Abstract. This study focuses on the impacts of climate changes by applying a conventional general circulation model (HadCM3) to report the output subscale of LARS-WG using HadCM3 statistical model. We have used SWAT model to study the changes in the runoff volumes of the Chehel-Chai basin. The study results suggest regardless of the occurrence of each of the three future rounds, the temperature would rise causing an increase in rainfall. This would cause a decrease in the rate of flow of Chehel-Chai River. An increase in evaporation, transpiration, and discharge of the river water for irrigation would subsequently reduce the flow rate of the Chehel-Chai River.
Keywords: *HadCM3 model, statistical models LARS-WG, SWAT*

Introduction

Climate changes are known to cause alteration in the duration, intensity, form, and timing of rainfall in many areas. Consequently, droughts and flooding would be immanent in the nearby areas. On the other hand, water resource management programs have been affected by the rapid climate changes more often in the recent years. Since 1995 numerous research and modeling studies have focused on forecasting the impacts of rapid climate changes on water resource plans. As large-scale global climate models are based on the defined networks (150 to 300 km) they would not be applied directly to assess the hydrological models.

Among a few detailed models one might identify the Atmospheric-ocean general circulation models coupled type (AOGCM). The model is designed and developed at the Hadley Centre, UK. The resolution of this model is 2.75 by 3.75 degrees (IPCC, 2007). The model is described by (Gordon et al., 2000; Pope et al., 2000). HadCM3 consists of two components, namely Oceanic and Atmospheric HadAM3 (atmospheric model) and HadOM3 (the ocean), which has an ice-sea model. This model requires the setting surface flux. Based on a calendar year (365 days and 30 days of month) this model has a high resolution oceanic components. Another advantage of this model is its good coordination between the atmosphere and the ocean.

Steele-Dunne et al. (2008) examined impact of climate change on the hydrology of river flow using ECHAM 5 general circulation models and emission scenarios A1B. By application of the conceptual rainfall-runoff model HBV-Light, they were able to evaluate the future river flow on 9 basins located in Ireland. Varanou et al. (2003)

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examined changes of quantity and quality of runoff in a basin in central Greece using SWAT model to show the reduction of flow as well as the increase of the nitrogen concentration.

As Yu et al. (2002), Chen et al. (2011) and Dhar and Mazumdar (2009) show the effect of climate change on water resources would be an increase in temperature for prolonged periods of time along with the impact of the increased CO_2 on the river runoff volumes in several regions drastic changes in daily rainfall;

Studies elsewhere have shown that rapid climate changes would also impact plant growth, irrigation time, and runoff volumes (Ficklin et al., 2009).

In a study in Taiwan Lin et al. (2010) developed an annual warning indicator to assess the impact of climate change on water resources. The study shows the annual changes impose significant impacts on the ground water system by changing the temperature, precipitation, as well as other variables such as evapotranspiration.

Koutroulis et al. (2013) studied the impact of climate change on a regional Greek water resource. They have concluded that owed it is necessary to improve and update local water management planning and adaptation strategies in order to secure future water supplies.

Nkhonjera (2017) provided a review about Understanding the impact of climate change on the dwindling water resources of South Africa. In general, the factors such as lack of political will, climate modelling constraints, and uncertainties in both climate and hydrological modelling are of particular importance that they feature highly in climate change impact analysis studies. Results showed diversity in climate model simulations should be seen as a positive attribute and a judicious selection, configuration, and calibration of these hydrological models need to reduce uncertainties in hydrological modeling.

Inouye et al. (2017) assessed parameters of Climate Impacts on water Resources in Idaho. A new scenario was developed to assess the range of impacts by developing a model of the river basin and two another quantitative models (a system dynamics model and a spatially-explicit integrated model) in order to explore research.

Seiller et al. (2017) studied the Influence of three common calibration metrics on the diagnosis of climate change impacts on water resource. In that investigation, based on thirty-seven Canadian catchments, three common objective functions (focusing on different sections of time series), and twelve lumped conceptual hydrologic models, aim to provide insight on this specific contribution to the uncertainty cascade. Results from this study show that the choice of an objective function, between pairs of common options, can clearly affect our interpretation of the impacts of climate change on water resources. Furthermore, these conclusions are dependent on the hydrologic model, illustrating the need for water managers to evaluate their operated model and studied catchments to appraise the predictability of their hydrological system and estimate their confidence level.

Nepal (2016) assessed the Impacts of climate change on the hydrological regime of the Koshi River. The results suggested that snowfall pattern, snowmelt, total discharge, and evapotranspiration are all sensitive to climate change. The results should be taken as indicative of how the hydrological regime might change in future rather than definitive. By incorporating high resolution data and replicating studies in reference catchments in the Himalayan region, analyses of this type can be made more reliable.

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The Study Area

The Chehel-Chai basin, with an area of 25.683 hectares is located at the 55º 23́-55º 38́ longitude and 36º 59́- 37º 13́ latitude. As a subdivision of the great Gorganrood basin, this basin is located within the city boundary of Minoodasht in Golestan Province and has a minimum and maximum elevation of 190 m and 2570 m above the sea level. *Fig. 1* shows the location of Chehel-Chai basin.

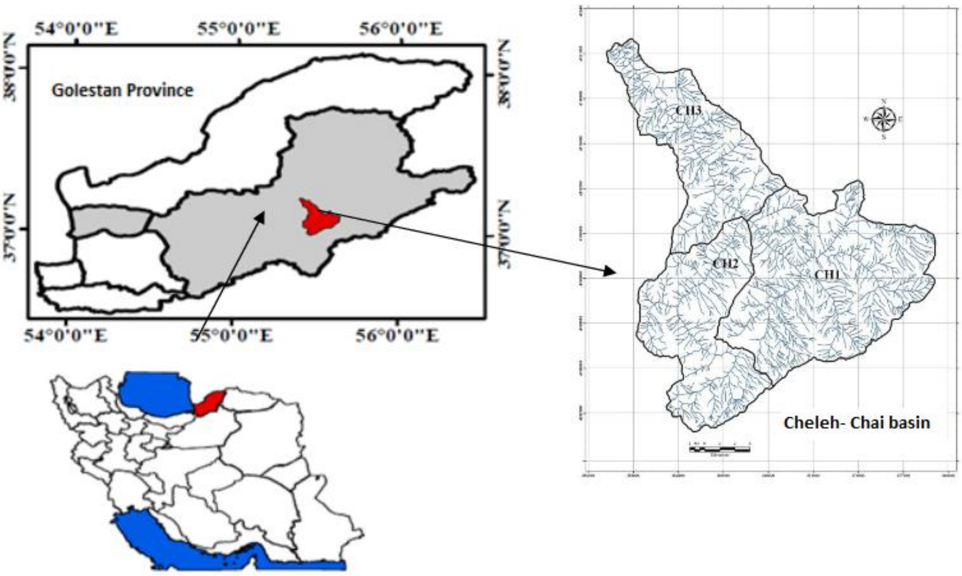

Figure 1. *Location map of Chehel-Chai basin in Golestan Province of Iran*

The Model

In order to gather the runoff data of the study area as one of the main inputs of the model, we used the DEM map, the land use map, and the soil map, (*Figs. 2-4* respectively).The meteorological data was collected and prepared in ArcGIS 10.3 for entry into the model. We used also the moisture content, saturated hydraulic conductivity, and soil bulk density based on the available data. The CROPWAT data were obtained using the available database of the soil texture.

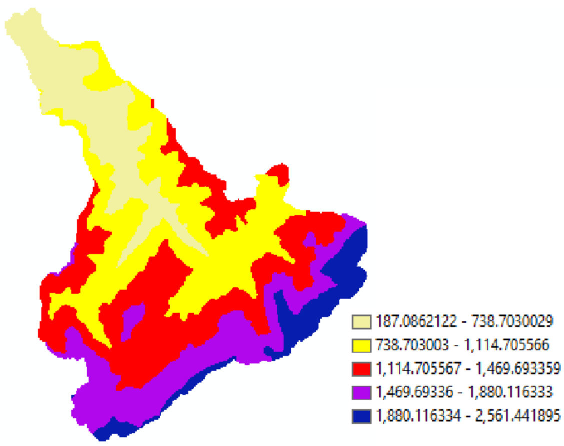

Figure 2. *Map of digital elevation model (DEM) at Chehel-Chai basin*

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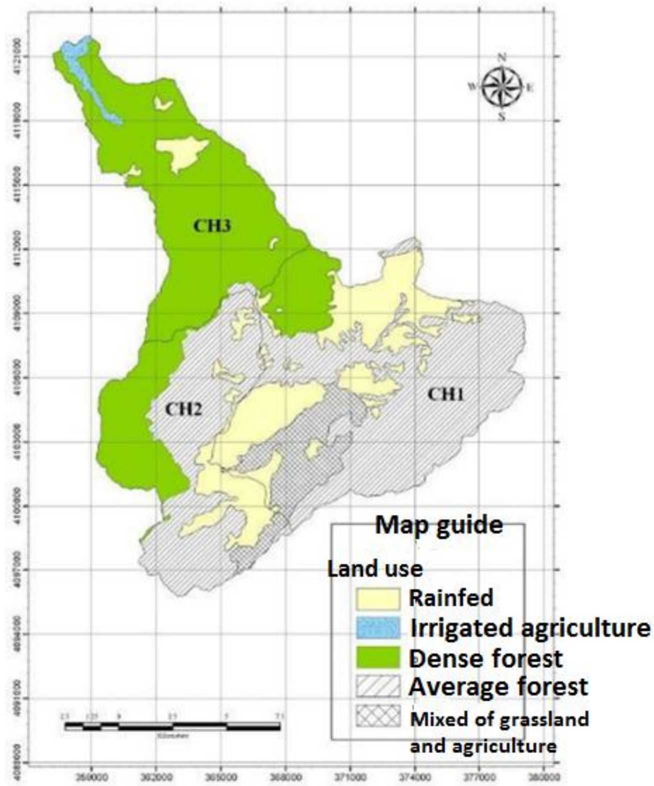

Figure 3. *Land use map of Chehel-Chai basin*

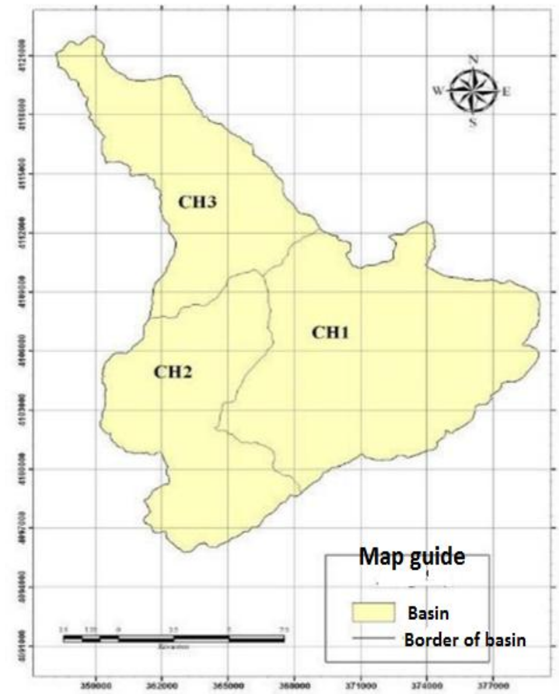

Figure 4. *Map of soil hydrological groups of Chehel-Chai basin*

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The minimum and maximum monthly temperature ranges of the study area are provided in *Figs. 5* and *6* respectively. *Table 1* shows other physiographic parameters used in the model.

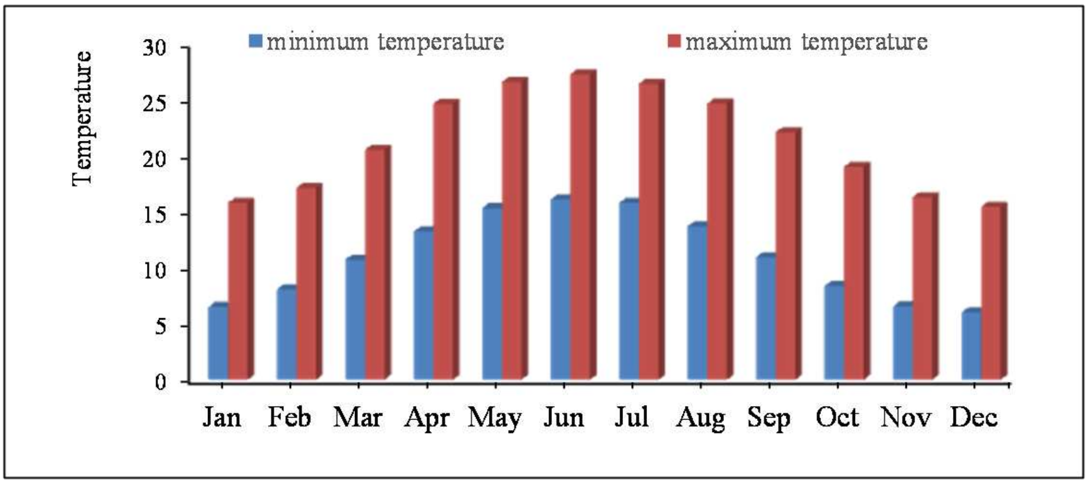

Figure 5. *The minimum and maximum air temperature at Chehel-Chai evaporation measuring stations*

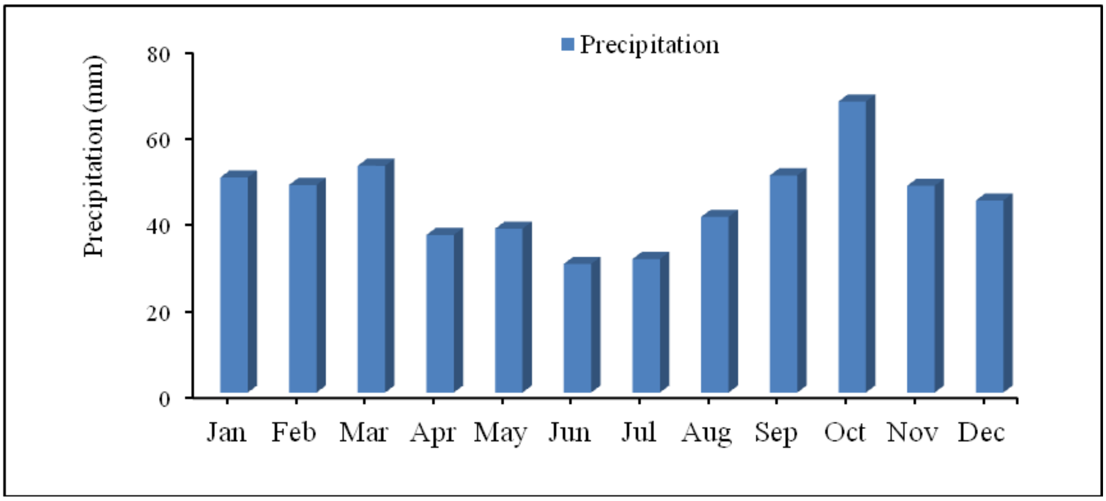

Figure 6. *monthly precipitation of Chehel-Chai evaporation measuring stations*

Table 1. *Chehel-Chai sub-basins physiographic characteristics*

Row	Sub-basin	Area	Perimeter	Weighted	Weighted
1	CH1	118.82	53.89	46.89	1388.80
2	CH2	71.26	45.99	42.88	1327.31
3	CH3	66.75	46.19	47.06	713.56
Total		256.83	95.24	45.82	951.14

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HadCM3 Climate models

In statistical method of determining optimal function, large-scale climate variables have been simulated by general circulation models in future periods, these functions are applied as input variables in the surface. In this method, a statistical relationship using simple regression, multivariate, neural networks and stations between actual behavior and general circulation model output is generated. After verification, these equations can be used in downscaling projections of future emission scenarios. In this study, in order to study climate change from a traditional to HadCM3 general circulation models and downscaling to weather generator LARS-WG statistical model was used.

LARS-WG for simulation of wet and dry periods, rainfall and solar radiation, distribution of the quasi-experimental uses the following form:

$$EPM = \{a_0, a_i, h_i, i = 1, \ldots, 10\} \quad \text{(Eq.1)}$$

That histogram consists of 10 floors and each floor is defined in $[a_{i-1}, a_i)$ ed, so $a_{i-1}<a_i$ and h_i frequency of observed phenomena, i*th* is on the floor. In this research initially to calibrate and verify models and scenarios, the criteria NSE, RMSE and R2 were used. Followings are the relations for the presented criteria:

$$NSE = 1 - \frac{\sum_{i=1}^{N}(O_i - S_i)^2}{\sum_{i=1}^{N}(O_i - \bar{O})^2} \quad \text{(Eq.2)}$$

$$RMSE = \left[\frac{1}{N}\sum_{i=1}^{N}(S_i - O_i)\right]^{1/2} \quad \text{(Eq.3)}$$

$$R^2 = \left[\frac{\frac{1}{n}\sum_{m=1}^{n}(S_i - \bar{S})(O_i - \bar{O})}{\sigma_s \times \sigma_o}\right]^2 \quad \text{(Eq.4)}$$

where: O_i: observational data, S_i: Estimated data, $\bar{\mathbf{O}}$ and $\bar{\mathbf{S}}$ observed data and estimated mean, σ is the variance.

First, LARS-WG model was calibrated for the base period 1982-2010, and then by restarting the calibrated model, the validation was used for the base period. The results of calibration and verification data and the observed small-scale temperature and precipitation are presented in *Table 2*. According to Nash-Sutcliffe coefficient of determination and capability indices, the model has a good capability in down-scaling the regional temperature data and the region precipitation in future periods. After securing the model simulation results, the maximum temperature variables (*Fig. 7*) and minimal (*Fig. 8*) and precipitation (*Fig. 9*) by HADCM3 model for future periods from 2011 to 2030, 2046 to 2065 and 2080 to 2099 during climate scenarios SRA1B, SRA2 and SAB1 were predicted.

Table 2. *Statistics error of climate parameters observed and modeled by model LARS-WG*

Monthly maximum temperature average			**Monthly minimum temperature average**			**Monthly average precipitation**			**Period**
R^2	RMSE	NSE	R^2	RMSE	NSE	R^2	RMSE	NSE	
0.99	0.49	0.99	0.99	0.38	0.99	0.88	5.2	0.80	Validation
0.99	0.54	0.98	0.99	0.43	0.99	0.79	6.8	0.73	Calibration

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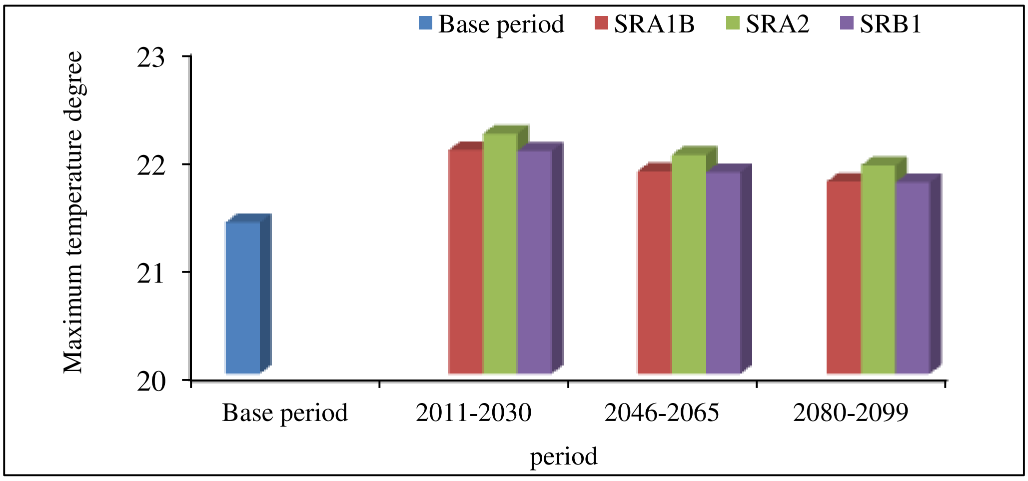

Figure 7. *Long-term average annual maximum temperature for the base period and future*

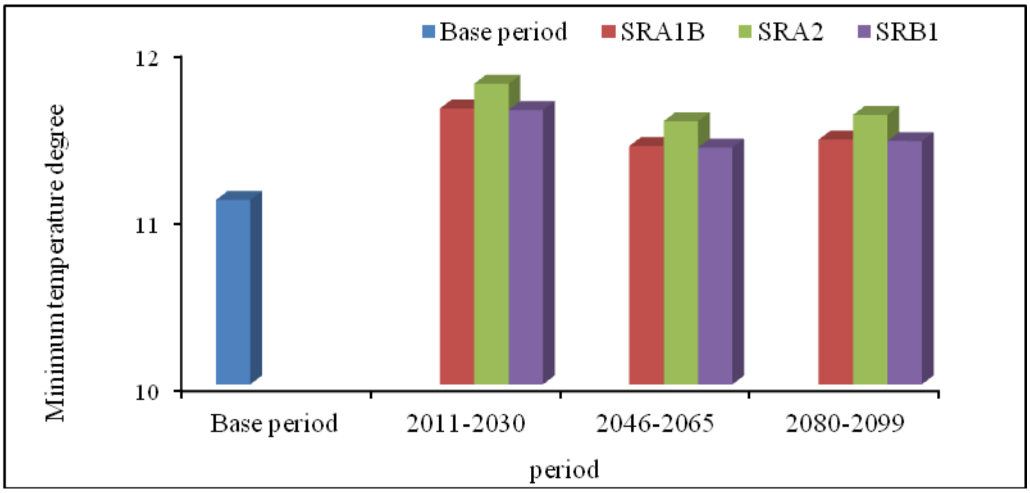

Figure 8. *Long-term average annual minimum temperature for the base period and future periods*

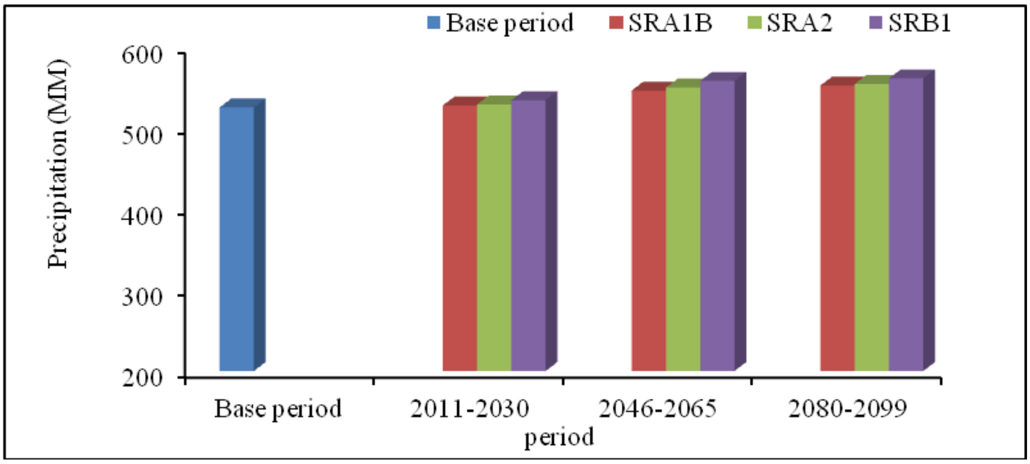

Figure 9. *The long-term average annual precipitation for the base period and future periods*

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By comparing the average values of the maximum temperature specified in *Fig. 7* it is obvious that the maximum temperature in all three rounds will be higher than the base period. (Rise in temperature to 0.82 degrees' Celsius maximum temperature 0.37) In all three scenarios the highest maximum temperature will occur in 2011-2030 period, however, for future periods, during the period 2080-2099 all three scenarios are lower than the maximum temperature will be two periods of 2011-2030 and 2046-2065.

In *Fig. 8*, the minimum temperature in all three rounds will be higher than the base period. (0.31 to 0.70 degrees' Celsius rise in temperature, at least) In all three scenarios the maximum temperature will occur at least in the period 2011-2030, however, for future periods, during the period 2046-2065 all three scenarios are lower than the minimum temperature in two periods 2011-2030 and 2080-2099.

In *Fig. 9* by comparing the average values of rainfall it is clear that precipitation in all three rounds will be higher than the base period (Increased precipitation between 2.34 to 35.89 mm) and rainfall amounts will increase over time. In all three scenarios, the most precipitation will occur over the period 2080-2099, however, for future periods, during the period 2011-2030 all three scenarios are less than two periods 2046-2065 and 2080-2099.

Flow simulation using the SWAT

SWAT distribution model was used to assess the impact of climate change on hydrology of Chehel-Chai basin. This model is sensitive to changes of Earth and climate. Hydrological cycle, which in this simulation model is based on water balance equation is as follows:

$$\mathrm{SWt} = SWo + \sum_{i=1}^{t}(Rday - Qsurf - Ea - Wseep - Qgw) \qquad \text{(Eq.5)}$$

In this regard SW_t is final amount of water in the soil (mm), t- time (days), SW_o – the amount of water in the soil (mm), R_{day}- amount of precipitation in day i (mm), Q_{surf}- amount of surface runoff in day i (mm), E_a- evapotranspiration in day i (mm), W_{seep}- amount of water in the soil profile penetrated in the cortical area (mm) and Q_{gw}- flow back in the day i (mm)

In this regard, after the gathering and preparation of needed meteorological data and maps, according to the initial values of the parameters, SWAT continuous rainfall-runoff model for the base period of the years 1982-2010 was calibrated and validated. Additional SWAT CUP was used for model calibration software. During the study period to simulate the runoff of years (2000-1982) to calibrate the model and year period (2010-2001) were selected for validation period. Then, the effects of climate change and global warming on the hydrological basin in future periods were evaluated.

In simulation using the model it is necessary to indicate the parameters that output of the model shows the most sensitivity and for the calibration of the model, the sensitive parameter should only be used. For this purpose, after the initial implementation of the model to analyze the sensitivity of the effect of various parameters on model outputs was based on objective function of Nash-Sutcliffe.

This software with the observed data and parameters to modify, the changes range for each parameter identified and covered most of the measured data, the minimum thickness of the strip uncertainties may follow. *Table 3* shows the results of a sensitivity analysis to calibrate and validate in SWAT CUP. Also performance evaluation model

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using coefficients R2, NS, RMSE, P-factor and R-factor between observed and simulated data was performed (*Table 4*).

Table 3. *Optimal values of the parameters studied for runoff simulation with the range of parameter values.*

Maximum	Minimum	Optimal value	Name of parameter	Row
1.01	0.27	0.83	R__CN2.mgt	1
116.47	58.71	93.74	V__CANMX.hru	1
0.65	0.34	0.58	V__ESCO.hru	3
0.25	-0.25	0	V__ALPHA_BNK.rte	4
1.25	0.69	0.74	R__HRU_SLP.hru	5
9.79	2.47	2.72	V__SFTMP.bsn	6
0.31	-3.42	0.02	V__SMTMP.bsn	7
49.64	3.55	24.82	R__TLAPS.sub	8
0.14	0.02	0.05	V__GW_REVAP.gw	9
0.46	0.06	0.20	V__RCHRG_DP.gw	10

Table 4. *The resulting coefficient for calibration of the model to simulate runoff monthly*

R-factor	P-factor	RMSE	NS	R^2
1.07	0.84	0.175	0.66	0.71

Fig. 10 shows simulated and observed values with 95% confidence interval for calibration periods. According to the results of the calibration, the model has a good ability to simulate water flow in the time period.

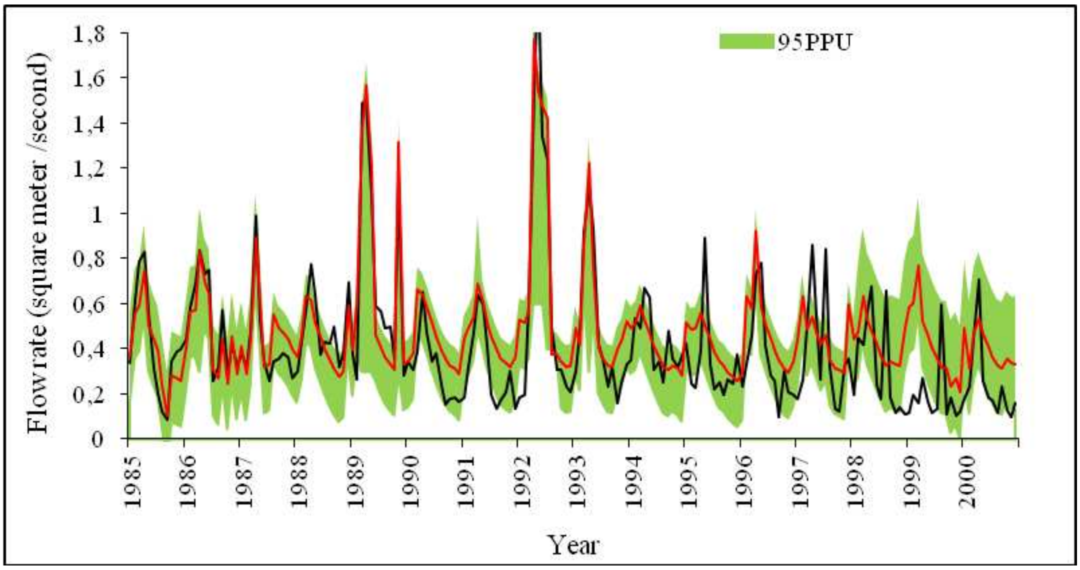

Figure 10. *Simulated and observed values with the confidence interval for calibration periods*

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In the calibration, results in 95% confidence level for the validation process is shown in *Fig. 11*. Amounts related to the accuracy of model is provided in *Table 5*, which indicates the proper functioning of the model.

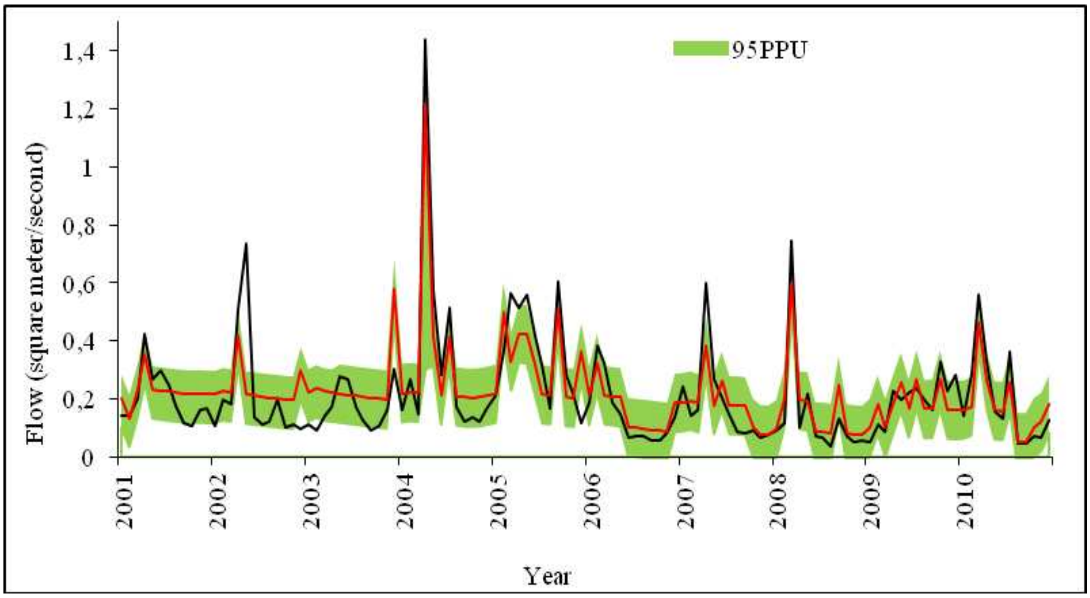

Figure 11. *Simulated and observed values with the confidence interval for validation period*

Table 5. *Coefficients obtained in validation process model to simulate the monthly runoff at the base time*

R-factor	P-factor	RMSE	NS	R^2
1.21	0.71	0.117	0.61	0.69

After calculating average temperatures, minimum, maximum, radiation and precipitation, the effect of climate on Chehel-Chai basin was found. Then the model for the base period of continuous rainfall-runoff SWAT was calibrated and validated. In the next step to assess the impact of climate change on runoff was investigated. Given that situation in the future time series (under the impact of climate change) the objectives of this study was, therefore, to obtain the time series , daily meteorological parameters related to SWAT model was replaced in future and by applying the calibration parameters in the base period was predicted for time series period. River flow scenarios predicted values over AIB, A2 and B1 in the period 2011 to 2030 (*Fig. 12*), 2046-2065 (*Fig. 13*) and 2080-2099 (*Fig. 14*) is provided.

Variable rate long-term average for the base period (1982 to 2010) and future periods (2011 to 2030, 2046 to 2065 and 2080 to 2099) during climate scenarios SRA1B, SRA2 and SRB1 is presented in *Table 6*.

In all three scenarios the highest rate in the period 2080-2099 will occur in future periods, however, for future periods, during the period 2046-2065 all three scenarios rate less than two periods, 2011-2030 and 2080-2099.

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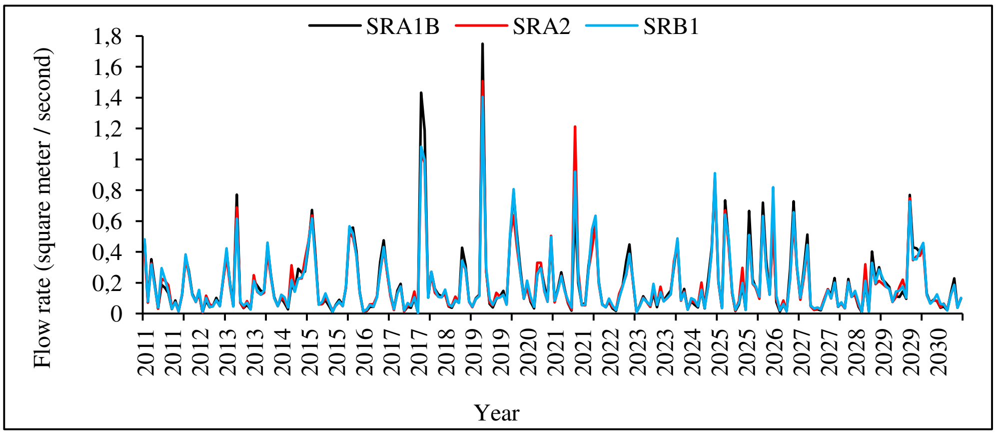

***Figure 12.** The predicted values river flows in Horizon 2020 (during 2011-2030) in scenarios AIB, A2 and B1*

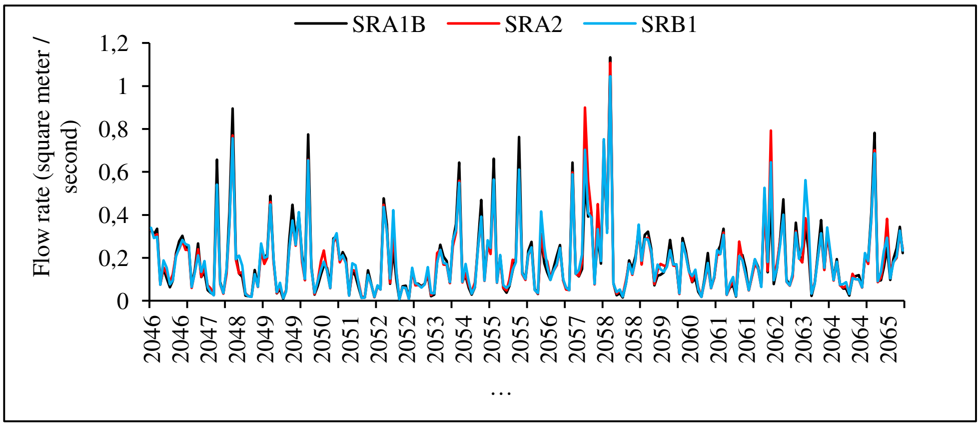

***Figure 13.** The predicted values river flows in Horizon 2055 (during 2046-2065) in scenarios AIB, A2 and B1*

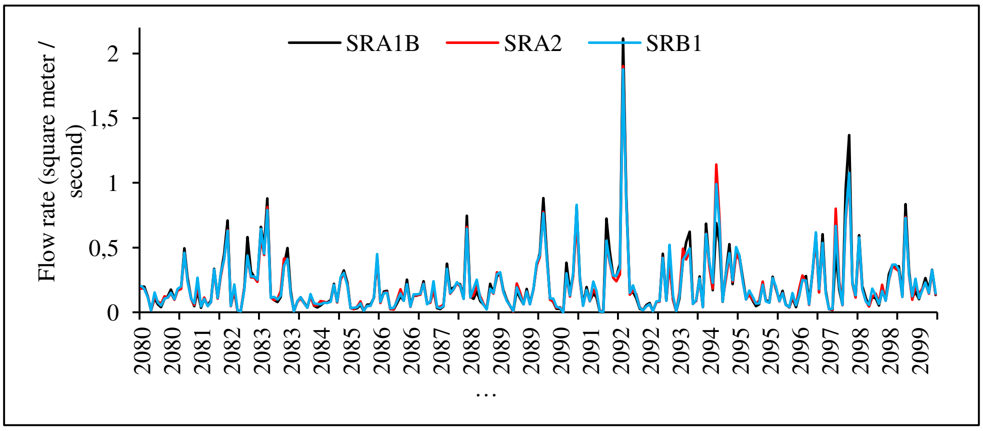

***Figure 14.** The predicted values river flows in Horizon 2090 (during 2080-2099) in scenarios AIB, A2 and B1*

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Table 6. *Average long-term variable annual rate for the base period and future periods*

Long-term average of flow	Scenario	Period
0.339	-	base
0.199	SRA1B	2011-2030
0.192	SRA2	
0.195	SAB1	
0.188	SRA1B	2046-2065
0.186	SRA2	
0.189	SAB1	
0.217	SRA1B	2080-2099
0.209	SRA2	
0.213	SRB1	

Conclusion

Assessment of climate change impacts on water resources is carried out in two steps. In the first step changes in temperature, rainfall was investigated in the second phase the change in runoff by SWAT model output step was carried out. Results are as follows:

- According to the approved model LARS-WG, climate change was for future periods from 2011 to 2030, 2046 to 2065 and 2080 to 2099 during climate scenarios SRA1B, SRA2 and SRB1 separately for variables minimum temperature, maximum temperature and precipitation. By comparing the values of the average minimum temperature is specified that the minimum temperature in all three rounds will be higher than the base period. (0.31 to 0.70 ° C rise in temperature) In all three scenarios, the maximum temperature will occur at least in the period 2011-2030; however, for future periods, during the period 2046-2065 all three scenarios are lower than the minimum temperature two periods 2011-2030 and 2080-2099.
- By comparing the average values of maximum temperature, specified that the maximum temperature in all three rounds will be higher than the base period. (Rise in temperature to 0.82 degrees Celsius 0.37) In all three scenarios the highest maximum temperature will occur in 2011-2030 period; however, for future periods, during the period 2080-2099 all three scenarios are lower than the maximum temperature and will be two periods of 2011-2030 and 2046-2065. Overall results indicated that in future climate scenarios, rising temperatures will occur. In research Yu et al. (2002) also shows an increase in temperature results in prolonged periods of time and drastic changes in daily rainfall events is likely.
- Comparing the average values of rainfall, it was found that precipitation in all three rounds will be higher than the base period. (Increased precipitation between 2.34 to 35.89 mm) and with rainfall amounts will increase over time. In all three scenarios, the most precipitation will occur over the period 2080-2099; however, for future periods, during the period 2011-2030 all three scenarios is less than two periods 2046-2065 and 2080-2099.
- After the initial login parameters SWAT model and its implementation, an analysis of the sensitivity of the effect of various parameters on model outputs, Nash-Sutcliffe based on the objective function has little effect in output

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parameters that have to be removed and only be used for model calibration and sensitivity parameters. As a result, the order of 10 parameters including number curve, soil evaporation coefficient, the coefficient α to save waterborne Channel coast, the rate of temperature change with height in the suburbs, the average temperature for the rain turning to snow, the temperature of melting snow, the rate of change of temperature with height, coefficient of penetration determination to deep aquifer and feeding percentage of deep groundwater from shallow aquifers showed the greatest sensitivity. These results clearly show that the number of curves that are associated with land use change, are most susceptible to runoff.

- After the calibration and validation of models based on super critical parameters, performance evaluation model using coefficients R^2, NS, RMSE, P-factor and R-factor between observed and data was simulated. The results in this section in calibration and validation step indicated that the model efficiency coefficient (Nash-Sutcliffe) was positive and correlation coefficient was greater than 0.6 and RMSE value was too low. Therefore, this model has a good ability to simulate water flow in the time period.
- Comparing the long-term average annual rate, it is clear that all three rates in the future will be less than the base period (reduction rate of 0.122 to 0.153 cubic meters per second). In all three scenarios the highest rate in the period 2080-2099 will occur in future periods, however, for future periods, during the period 2046-2065 all three scenarios rate less than two periods, 2011-2030 and 2080-2099. Varanou et al. (2003) research using the SWAT model observed that the current decline will occur in the future. Although research results (Ficklin et al., 2009) showed that the precipitation and evapotranspiration are decreased, but it seems to be the opposite of Chehel-Chai River basin. So that the results showed that temperature and precipitation and runoff is reduced. Despite the increased rainfall, rising temperatures will lead to increased evapotranspiration, which will lead to reduced flow in rivers. However, the issue of excessive exploitation from water sources can be known related.

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CHANGES IN OENOCLIMATE APTITUDE INDEX CHARACTERIZING CLIMATE SUITABILITY OF ROMANIAN WINE GROWING REGIONS

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Abstract. The oenoclimate aptitude index (IAOe) reveals climate suitability for wine production in temperate climate conditions. For Romanian wine regions its values were quite stable throughout the years, indicating the possibility of practising certain types of wine production. In the last decades the IAOe values have increased. By using gridded data at 0.1 degrees resolution of temperature, precipitation and sunshine duration for the entire Romanian territory, we calculated the IAOe averages for the 1961-1990 and 1991-2013 periods for 50 wine-growing centers which are representative for the Romanian viticulture. The results indicate that the IAOe mean at Romanian viticulture area level is currently of 4629 units, 270 units higher than the ones between 1961-1990. Due to this increase, the IAOe averages for the wine-growing centers from Moldova, Muntenia, Banat, Crișana and Southern regions have passed during the recent decades to values corresponding to an upper suitability class for wine production. In the wine-growing centers from Transylvania, the IAOe maintains in the range of suitability for white table wines, while for the ones from Dobrogea and Danube Terraces the IAOe maintains in the range of suitability for quality red wines. The study provides the scientific support needed for the re-evaluation of the wine type production practiced within Romanian wine growing-centers.
Keywords: *viticulture, wine production, Transylvania Plateau, Moldavian Hills, suitability, spatial distribution*

Introduction

The study of bioclimatic indices characterizing wine regions across the globe led to the conclusion that they have been modified by climate change in the last decades (Jones et al., 2005; Duchene and Schneider, 2005; Gatto et al., 2009; Beltrando and Briche, 2010; Kryza et al., 2015). These changes are associated with changes in grapes and wine composition and characteristics (Duchene and Schneider, 2005; White et al., 2006; Laget et al., 2008; Ramos et al., 2008). Forecasts on vineyards climate predict major shifts in the distribution and structure of viticultural areas worldwide in the coming decades (Hannah et al., 2013; Moriondo et al., 2013). In this context, knowing the impact of climate change on the Romanian viticulture (fifth place in Europe as vine area and thirteenth in the world as wine production; OIV, 2016), becomes mandatory in order to develop strategies for its adaptation to climate change.

Climate change manifests globally for about 50 years by increasing temperatures and sunshine duration amid relative stability of rainfall, accompanied by aridity and intensification of weather extremes (Beniston and Tol, 1998; Karl, 1998; Fraederich et

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al., 2001; Thomas et al., 2008; Wild, 2012; IPCC, 2013). In Romania this phenomenon has been widely analysed in the literature of the past decades. The main observed developments are: significant increases in temperature and insolation during the winter, spring and summer, accompanied by constant rainfall during the year (Dumitrescu et al., 2014); significant increases in maximum and minimum temperatures during the year and unclear trends in precipitation evolution (Busuioc et al., 2015). Similar trends were observed at regional level (Piticari and Ristoiu, 2012; Planchon and Endlicher, 2014).

These climate developments has caused changes to the Romanian wine regions level as well: in the vineyards in the northern half of the country, which are cooler and specialized in white wines production, red wine grape varieties have started to be grown, while in those in the southern half, which are warmer and specialized in red wine production, musts are lacking in acidity and rich in sugar accumulations. In spite of all this evidence to which results of some recent studies can be added (Bucur and Dejeu, 2016; Irimia et al., 2017) the impact of climate change on the Romanian viticulture is still little known. Therefore, the aim of this study is to reveal the changes in climatic conditions for wine production in the Romanian wine regions as a result of climate change.

Tin order to study climate characteristics of wine growing regions, a variety of bioclimatic indices are used. Many of them are heat summation indices: Winkler Index (WI; Amerine and Winkler, 1944), Huglin Index (HI; Huglin, 1978), average growing season temperature (AvGST; Jones, 2006). Others are complex indices such as: Latitude Temperature Index (LTI; Kenny and Harrison, 1992), Composite index (CompI; Malheiro et al., 2010) or combinations of thermal and hydrologic indices (Moriondo et al., 2013). As the heat summation indices do not show the entire complexity of climatic conditions that determine the suitability for wine production (Moriondo et al., 2013), and the complex indices mentioned above were developed for climate conditions not necessarily similar to temperate climate conditions characterizing Romanian viticulture, we carried out this analysis by using the *oenoclimate aptitude index* (IAOe) which was developed in Romania by Teodorescu et al. (1987). The IAOe is highly effective in representing climate suitability for wine production in temperate climate conditions (Irimia et al., 2013, 2014). Its efficiency is given by the fact that it integrates three climatic determinants of grapes and wine quality: temperature, precipitation and sunshine duration. Due to its efficiency, in the 80s the IAOe was used to zone Romanian viticulture (Teodorescu et al., 1987), while today it is largely used to analyse vineyards climate suitability for certain wine grape assortments.

According to the research results published so far, no study has yet been carried out in Romania to reveal changes appeared in vineyards climate suitability at the national level in the last decades. Some research reveal trends in extreme weather conditions in Romanian viticulture (Bucur and Dejeu, 2016) or shifts in climate suitability for some wine regions (Irimia et al., 2017). This study aims to provide the current IAOe values revealing current climate suitability for the wine production for the 50 most important Romanian wine growing centers, spread all over the Romanian wine growing regions. The current IAOe values could offer these 50 wine growing regions the scientific support for their adaptation to the new climatic context.

The study is structured as follows: determination of spatial distribution and suitability for the wine production of the IAOe between 1961-1990 and 1991-2013; analysis of changes in the IAOe averages and suitability at the wine growing centres level; identification of causes of IAOe increases.

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Materials and methods

Study area

Romania is located between 43°37'- 48°15' N lat. and 20°15'-29° 44' E long (*Figure 1*) and has a temperate continental climate, Dfb and Dfa in Koppen-Geiger climate classification (Kottek et al., 2006). Romania’s landscape is complex and diversified, with 28% represented by mountains; 42% represented by plateaus and hills; and 30% represented by plains (Bălteanu et al., 2010).

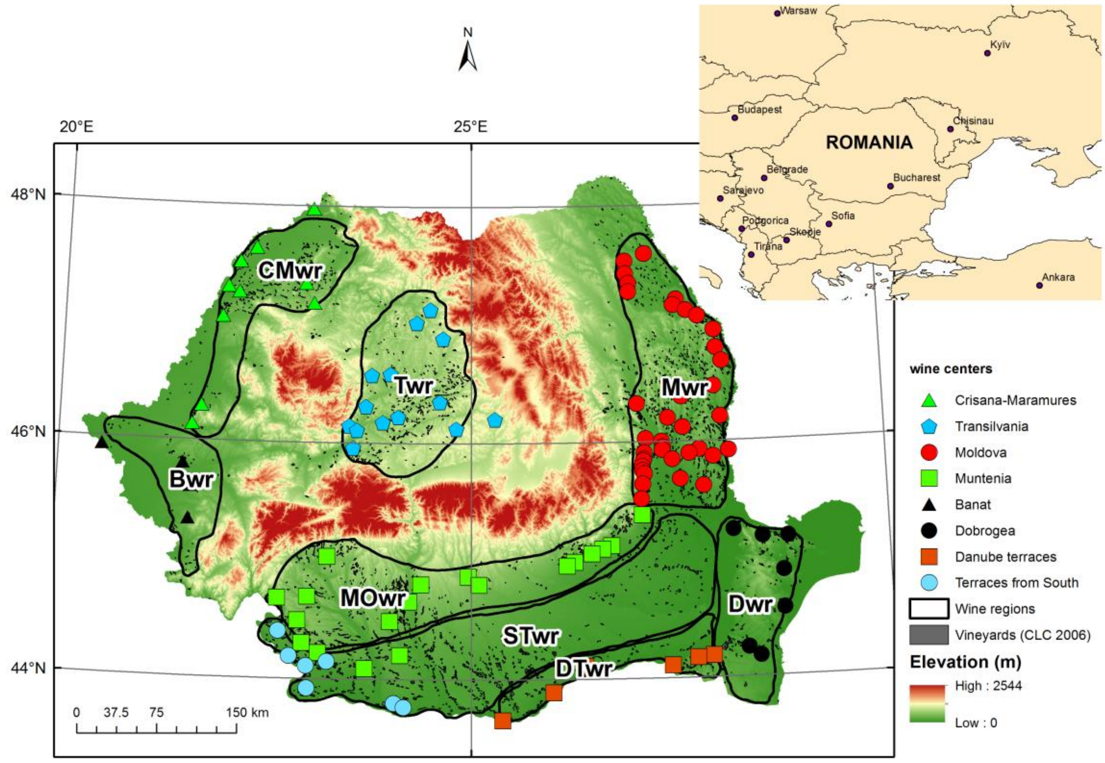

Figure 1. *Map of Romanian wine regions and wine growing centers*

As a viticulture country Romania has 197,000 ha vine plantations and produces about 6 mil Hl wine/yr (OIV, 2016). Romanian viticulture area is structured in eight large wine regions, corresponding to the historic provinces of the country. They are (*Figure 1*): Transylvanian Plateau Wine Region (Twr); Moldavian Hills Wine Region (Mwr); Muntenia and Oltenia Hills Wine Region (MOwr); Banat Hills Wine Region (Bwr); Crişana and Maramureş Hills Wine Region (CMwr); Dobrogea Hills Wine Region (Dwr); Danube Teraces Wine Region (DTwr); Sands and other suitable terrains from the South Wine Region (STwr). These eight large wine regions encompasses 141 wine growing centres (wine growing centre=vine plantations around a locality) belonging to 37 traditional wine regions. The study refers to 50 wine growing centers located in all eight large Romanian wine regions. For this study we used the map of the Romanian viticulture extracted from the Corine Land Cover Database 2006 Europe (EEA, 2006).

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Methods

The climatic data base used to calculate the IAOe values for the Romanian wine regions is represented by gridded data at 0.1 degrees resolution (10 x 10 km) of daily average temperature, annual precipitation and sunshine duration during the 1961-2013 extracted from the ROCADA database (Dumitrescu and Bîrsan, 2015). This database includes 9 climate parameters and it was achieved by interpolation of daily values recorded at meteorological stations throughout Romania by using the MISH software (Szentimrey and Bihari, 2007). The number of the meteorological stations used varies according to the climate parameter: 150 stations for air temperature, 188 stations for precipitations and 135 for sunshine duration (Dumitrescu and Bîrsan, 2015). In the first stage of the study the daily values were used to calculate the sums of active temperatures (Σta, °C/April 1st to September 30th), precipitation during the growing season (PP, mm/April 1st to September 30th) and actual sunshine duration (ASD, hour/April 1st to September 30th). In the second stage, these three parameters were used to compute the IAOe index at the same 0.1 degrees resolution (approximately 10×10 km). In the third stage the IAOe spatial data was downscaled at a finer resolution (100 x 100 m) by using the regression-kriging approach and the terrain altitude as predictor.

The IAOe is calculated for the April 1st to September 30th period according to Teodorescu et al. (1987) formula (Eq. 1):

$$IAOe = ASD + \Sigma t_a - (PP - 250) \qquad \text{(Eq.1)}$$

where: ASD = actual sunshine duration (hours, April 1st to September 30th), Σt_a = sum of daily average temperatures ≥ 10 °C (April 1st to September 30th), PP = precipitation (mm, April 1st to September 30th) and 250 = minimum precipitation needed for unirrigated vines (mm). For situations where PP < 250 mm, the IAOe is computed as the sum of ASD and Σt_a (Teodorescu et al., 1987).

In Romanian wine regions the IAOe varies between 3793 and > 5200 units, with values higher than 5200 units characterizing vineyards at the southern limit of Romania (43 °lat N) which produce mainly table grapes (Oşlobeanu et al., 1991). In this study, in order to identify the changes in climate suitability for wine production of the Romanian wine regions, the IAOe is classified according to Irimia et al. (2014) as follows: IAOe < 3793= unsuitable for grapevine growing; IAOe =3793-4300= suitable for white table wines, sparkling wines, wines for distillates (WTW+SW+WD); IAOe=4301-4600=suitable for quality white wines and red table wines (QWW+RTW); IAOe > 4600 = suitabile for quality red wines (QRW).

Results

Spatial distribution and the suitability for wine production of the IAOe between 1961 and 1990

The spatial distribution of the IAOe between 1961 and 1990 reflects the already known base climate suitability of Romanian wine regions, attested by their specific types of wine production (*Figure 2*): climate suitability for red quality wine in the southern half of the country where the STwr, DTwr, Dwr and MOwr are located; and climate suitability for white wines in the northern half of the country where Mwr, Twr, Bwr and CMwr are located.

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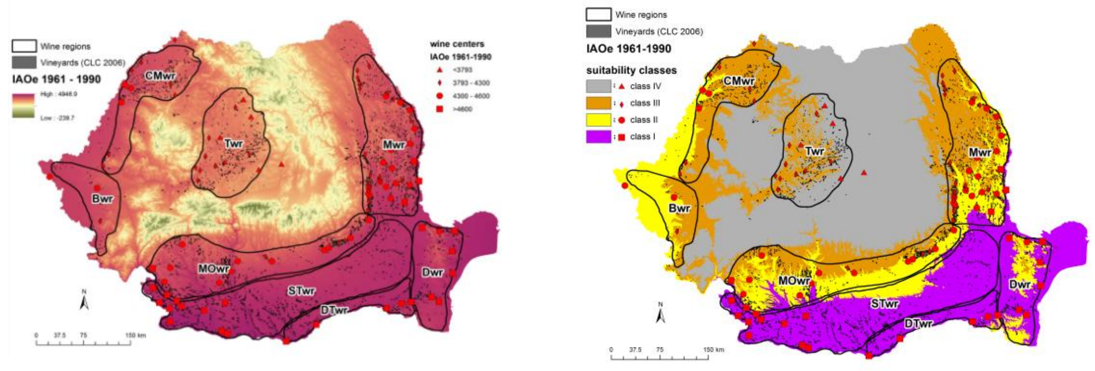

a. b.

Figure 2. *Spatial distribution of IAOe in Romania during the 1961 to 1990 time period and location of the main Romanian wine producing centers: a. continuous values; b. classified values. Values were calculated based on gridded data at 0.1 degree resolution of temperature, precipitation and sunshine duration extracted from ROCADA data base (Dumitrescu and Bârsan, 2015) and classified according to Irimia et al. (2014)*

Statistics for the spatial distribution of the IAOe across the Romanian wine regions (*Table 1*) show that between 1961-1990 its averages were between 3513 and 4647 units, with an average of about 4359 units indicating the prevailing climate suitability for QWW and RTW. The warmest wine regions and the most suitable for red wines are Dwr, DTwr and STwr where the IAOe averages exceed 4600 units (suitability for QRW); for all northern wine regions - Mwr, Twr, Bwr and CMwr - the IAOe averages are bellow 4300 units revealing suitability for white wine production (WTW, SW, WD); the IAOe for the MOwr, located at the transition zone between the southern and northern of Romania, has an average of 4300 units revealing suitability for QWW and RTW. IAOe Max reveals temporal variability of climate suitability of the wine regions specialized in white wine production, which in a number of years manage to produce even QRW. An exception is the Twr where the IAOe Max (4147 units) reveals the total lack of suitability for red wine production during the 1961 to 1990.

Table 1. *Statistics for the IAOe spatial distribution within Romanian wine regions during the 1961 to 1990 and during the 1991 to 2013*

Wine region	**Surface**	**Statistical parameters IAOe 1961-1990**				
	(Mln ha)	Min	Max	Range	Mean	STD
Twr	1,489	2815,3	4147	1332	3798,3	182,6
Mwr	1,949	2863,3	4714,3	1851	4249,0	222,1
MOwr	2,069	3041,5	4814	1772,5	4345,3	197,5
Bwr	0,726	3477,7	4471	993,3	4275,2	146,4
CMwr	1,121	3089,5	4383,4	1293,9	4134,8	168
Dwr	0,993	3870,9	4829,3	958,3	4625,1	130,7
DTwr	0,542	4480,7	4884,9	404,2	4736,4	62,5
STwr	2,933	4469,7	4937	467,3	4708,4	91,8
Average	**-**	**3513**	**4647**	**1134**	**4359**	**150**
		Statistical parameters IAOe 1991-2013				
Twr	1,489	3039.8	4447.6	1407.9	4011.9	186.3
Mwr	1,949	3132.3	5004.3	1871.9	4537.2	223.5
MOwr	2,069	3313.8	5071.6	1757.8	4599.2	204.1
Bwr	0,726	3761.3	4775.7	1014.4	4561.1	148.4

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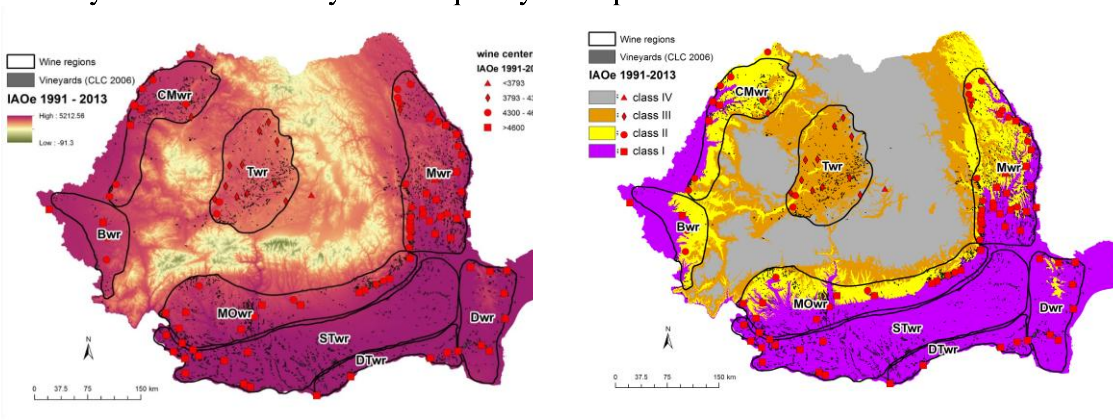
CMwr	1,121	3371.5	4702.5	1331.0	4446.4	169.1
Dwr	0,993	4125.8	5106.6	980.8	4896.6	137.2
DTwr	0,542	4761.5	5144.9	383.5	5005.6	60.2
STwr	2,933	4722.6	5203.1	480.5	4978.3	89.3
Average	**-**	**3778**	**4932**	**1153**	**4629**	**152**

Spatial distribution and suitability of the IAOe for wine production between 1991 and 2013

The spatial distribution of the IAOe between the 1991 to 2013 reveals major changes in climate suitability for wine production in Romanian wine regions (*Figure 3*): the area with IAOe exceeding 4600 units extended towards the northern half of Romania, within the area of Mwr, Bwr and CMwr while the southern wine regions are almost entirely covered by climate suitability for red quality wine production.



a. b.

Figure 3. *Spatial distribution of IAOe in Romania during the 1991 to 2013 time period: a. continuous values; b. classified values. IAOe values were calculated based on gridded data at 0.1 degree resolution of temperature, precipitation and sunshine duration extracted from ROCADA data base (Dumitrescu and Bârsan, 2015) and classified according to Irimia et al. (2014)*

Statistics for the spatial distribution of the IAOe between the 1991 to 2013 (*Table 1*) show that its mean for the Romanian wine regions increased during this time period by 270 units to 4629 units today. The current average reveals the predominance in Romania of climate suitability mainly for QRW and QWW in secondary. The IAO mean for the southern wine regions - STwr, DTwr and Dwr - remain suitable for QRW but it approaches by 5,000 units, value that characterize vineyards producing mainly table grapes. As a result of these increases in the wine regions from the northern half of Romania (Bwr, CMwr, Mwr) except the Twr, the IAOe exceeded between the 1991-2013 the threshold of 4300 units that indicates the suitability for QWW and RTW. Within the Twr, the coolest wine region of Romania, IAOe increased by 231 units but still lies in the range of suitability for WTW.

Changes in IAOe averages and suitability at the wine growing centers level

The data in *Table 2* shows that between 1961 and 2013, the IAOe mean increased by 236-346 units within Romanian wine growing centers. This increase causes major changes in climate suitability for wine production: except the wine centers from Twr,

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Dwr, TDwr and STwr, which still lie in the initial climate suitability class, the climate suitability of all the other wine growing centers from Romania passed to the upper suitability class: from class III to class II, and from class II to class I (*Table 2*). Although the IAOe average increased for each wine center in Twr, they still stands in the range of climate suitability for WTW, SW and WD. It is noteworthy here the obtaining of climate suitability for WTW, SW, WD by Bistrița, Batoş, Triteni and Apold wine growing centers of Twr, whose IAOe means during the 1961-1990 were below the threshold of suitability for WTW, SW and WD. In the case of southern wine centers of Romania (Dwr, TDwr and STwr) characterized even 1961 to 1990 by suitability for QRW, the IAOe average increased during the 1991 to 2013 to 5,000 units, a value specific to areas producing mainly table grapes.

The IAOe largest increase (346 units) during the 1991 to 2013 at Romania wine growing centers level is recorded for Lechinţa from Twr (*Table 2*) while at regional level for the W and NW of the country, within Teremia (+304 units), Măderat (+298 units), Diosig (+315 units), Şimleul Silvaniei (+315 units), Oradea (+323 units) and Zalău (+315 units) wine growing centers. IAOe important increases exceeding 300 units were recorded also within some wine growing centers from the central part of Mwr: Copou Iaşi (+303 units), Bucium (+312 units), Comarna (+320) and Murgeni (+312 units) are a few examples. IAOe increases have led to changes in climatic suitability for wine production in the affected areas.

The smallest IAOe increase at the country level (+238 units) is recorded in the Ştefăneşti wine growing center. However, the entire MOwr to which the Ștefănești wine growing centre belongs, is characterized by a low increase in IAOe average, of about +254 units. But in spite of these small increases many of the MOwr wine growing centers acquire climate suitability for QRW between 1991-2013 (*Table 2*).

Causes of IAOe increase

As shown in *Table 2* the cause of IAOe increase between the 1991 to 2013 is mainly the increase of active temperatures and of sunshine duration.

However, the largest increases in IAOe average recorded in Bistrița and Lechinţa wine growing centers from Twr and in certain wine growing centers from Mwr (Bucium, Comarna, Uricani, Copou, Vutcani) is caused by the increase of active temperatures and sunshine duration associated with a decrease of precipitation average. For the biggest part of Romanian wine growing centers, the IAOe increase is due exclusively to the increase of temperatures and insolation, while the precipitation amount is relatively unchanged.

The largest increase in the sum of active temperature during the 1991-2013 is recorded in the wine growing centers from Bwr and CMwr. It varies between +202...+221°C with an average of 212.5°C, as compared to +173…+216°C and an average of 193.78°C for the rest of Romanian wine growing centers. Significant increases in the sum of active temperatures impacting the IAOe averages are also recorded in some isolated wine growing centers as Bistrița and Lechinţa (+216 °C for both of them) from Twr; and also in some wine growing centers from the southern of Mwr (Tecuci, Iveşti, Corod) where the increase of the sum of active temperatures exceeded 200 °C.

Table 2. *Averages and differences for the oenoclimate aptitude index (IAOe), sum of active temperatures (Σt_a), precipitations during growing season (PP), and actual sunshine duration (ASD) for some Romanian wine centres, during the 1961-1990 and 1991-2013 time periods. Values were calculated based on gridded data at 0.1 degree resolution (10 × 10 km) of temperature, precipitation and sunshine duration extracted from ROCADA data base (Dumitrescu and Bârsan, 2015)*

Wine region	Wine growing center	IAOe/wine growing center (units)			Σt_a (°C)		PP (mm)		ASD (hours)	
		1961-1990	1991-2013	±	1961-1990	1991-2013	1961-1990	1991-2013	1961-1990	1991-2013
Twr	Bistrita	3689	4035	+346	2404	2620	420	396	1317	1423
	Lechinta	3892	4221	+329	2673	2889	409	398	1317	1419
	Batoș	3640	3956	+316	2580	2779	414	417	1321	1435
	Zagar	3866	4160	+294	2651	2841	416	430	1341	1444
	Jidvei	3983	4269	+286	2806	3000	400	417	1310	1427
	Sebeș	4034	4312	+278	2853	3058	395	412	1289	1368
	Apold	3680	3941	+261	2286	2465	437	436	1329	1414
Mwr	Hlipiceni	4218	4519	+301	2895	3093	388	388	1376	1480
	Cucuteni	3965	4230	+265	2716	2897	386	400	1353	1446
	Bohotin	4346	4653	+307	2981	3174	339	331	1430	1540
	Averesti	4155	4459	+304	2753	2929	339	335	1427	1537
	Husi	4436	4741	+305	2796	2971	322	322	1502	1617
	Nicoresti	4390	4677	+287	2968	3165	344	345	1447	1540
	Buciumeni	4274	4572	+298	2968	3165	351	350	1442	1535
	Panciu	4108	4397	+289	2932	3128	399	398	1376	1471
	Jaristea	4283	4567	+284	3074	3274	395	397	1362	1447
	Odobesti	4333	4614	+281	3074	3274	395	397	1362	1447
	Pechea	4602	4877	+275	3125	3320	302	312	1438	1517
	Cotesti	4244	4519	+275	3103	3302	395	397	1362	1447
MOwr	R. Sarat	4416	4690	+274	3151	3340	357	366	1420	1516
	Merei	4471	4753	+282	3202	3391	373	386	1427	1497
	Pietroasele	4066	4338	+272	3106	3294	373	386	1427	1497
	Tohani	4156	4401	+245	3200	3391	358	372	1407	1484
	Urlati	4398	4634	+236	2938	3128	385	396	1334	1400
	Topoloveni	4364	4604	+240	3004	3195	393	411	1464	1542
	Samburesti	4394	4646	+252	2919	3100	412	414	1466	1549
	Dragasani	4560	4819	+259	3172	3355	383	385	1462	1537
	Iancu Jianu	4595	4851	+256	3119	3306	358	365	1481	1558
	Segarcea	4767	5023	+256	3252	3435	310	321	1555	1642
Bwr	Teremia	4471	4775	+304	3110	3331	322	325	1440	1528
	Recas	4339	4626	+287	3041	3253	361	359	1408	1482
	Silagiu	4234	4524	+290	2971	3179	395	376	1414	1483
	Tirol	4276	4546	+270	2952	3154	413	415	1411	1484
CMwr	Halmeu	4172	4468	+296	2873	3084	392	409	1424	1527
	Valea lui Mihai	4288	4586	+298	2962	3170	346	365	1445	1544
	Diosig	4365	4671	+306	3018	3228	342	361	1451	1550
	Oradea	4334	4657	+323	2955	3167	338	335	1455	1550
	Saniob	4356	4671	+315	2949	3163	354	357	1436	1534
	Samsud	4099	4414	+315	2720	2938	395	403	1376	1483
	Maderat	4286	4598	+312	2968	3181	367	376	1459	1557
	Minis	4288	4573	+285	2988	3206	367	382	1441	1532
Dwr	Macin	4637	4913	+276	3203	3399	267	281	1513	1599
	Babadag	4747	5016	+269	3186	3382	212	224	1556	1650
	Murfatlar	4757	5025	+268	3145	3360	218	257	1534	1623
TDwr	Aliman	4792	5069	+277	3264	3464	260	290	1533	1648
	Oltina	4792	5064	+272	3232	3421	271	304	1544	1650
	Ostrov	4746	5010	+264	3220	3415	286	313	1515	1618
	Greaca	4668	4933	+265	3250	3450	329	340	1507	1586
STwr	Jiana	4716	5003	+287	3291	3481	313	314	1539	1633
	Dăbuleni	4853	5102	+249	3362	3535	295	301	1536	1617

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Precipitation during the growing season (PP) at Romanian wine growing centres level presents a relative stability (*Table 2*), with insignificant increases of about +1...+10 mm between the 1991 and 2013. Exceptions are the wine growing centers from Dwr where the average of PP during the 1991-2013 highly increases with values from +15 mm to + 39 mm. Increases of +16 mm to +30 mm of the PP are also recorded in the wine growing centers from Twr, in some wine growing centers from the eastern limit of MOwr (+15 mm...+20 mm), and in some wine growing centers from the northern half of CMwr (Halmeu Sanislău, Valea lui Mihai, Diosig). Conversely, the decrease in PP average between the 1991-2013 time period, ranging from -13 mm to -21 mm, are recorded in the wine growing centers from the central part of Mwr (Bucium, Uricani, Comarna, Copou) and in Bistrița and Lechința wine growing centers from the northern limit of Twr (-11 mm and -24 mm respectively.

The average of the sunshine duration (ASD) for the 1991-2013 time period increased by 60 to 180 hours for all wine growing centers from Romania. The highest values, higher than 100 hours, are recorded in the wine growing centers from Twr; in those within the northern half of Mwr; within the northern half of CMwr; and at the southern limit of Dwr (*Table 2*).

Discussion

By using spatial data of temperature, sunshine duration and precipitation for the entire Romanian territory at 0.1 degrees resolution (10×10 km), our study managed to provide the current IAOe averages for the most important 50 Romanian wine growing centers. The current IAOe averages reveal the current climate suitability for wine production of the wine growing centers, as it looks like after the climate change influence during the last two decades. Changes of the IAOe averages revealed by this study fall into developments of bioclimatic viticulture indices averages recorded at global and regional levels (Ramos et al., 2008; Laget et al., 2008; Duchene and Schneider, 2010), in the sense of increasing vineyards climate heliothermal resources amid diminishing water resources.

The general result of the study it the increase by 270 units of the IAOe average at Romanian wine growing regions level, from 4359 units between 1961-1990 to 4629 units between the 1991-2013. This increase shows the transition of the IAOe average for Romania viticulture from a range defining climate suitability for QWW (4300-4600 units) between 1991-2013 to a range defining the suitability for QRW (>4600 units) at present. The new values of IAOe calculated for the 1961 to 1990 are consistent with those reported for Romanian wine regions and wine growing centers by Teodorescu et al. (1987) and Oşlobeanu et al. (1991). They reflect accurately the traditional types of wine production specific to those areas: WTW, SW, DW, QWW in Twr, Mwr, CMwr and Bwr; RTW and QRW in Dwr, DTwr and STwr; QWW and QRW in MOwr located at the transition zone between the southern half and the northern half of Romania. Climate suitability evolution towards red wine production revealed by the current IAOe averages is consistent with that predicted by Mesterházy et al. (2014) and Stock et al. (2005) for temperate continental climate conditions in Europe. IAOe variability within Romanian wine growing regions maintains within the original range, although the higher frequency and levels of climatic extremes registered during the last decades (Bojariu et al., 2015), are able to influence the quality characteristics of grapes and wine production.

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IAOe averages for the 50 wine growing centers analysed in this study reveal the spatial variability of climate suitability within Romanian viticulture areas. They show the different abilities of wine growing centers to adapt to climate change, despite the overall environmental characteristics of the larger area they belong to. For example, the difference in the IAOe average for the contiguous Lechinţa and Batoş wine growing centers from the northern part of Twr is around 300 units. The same for the Sebes and Apold wine growing centers from Twr; for the Urlați and Tohani from MOwr; for the Silagiu and Recaş of Bwr; for the Valea lui Mihai and Diosig from CMwr. In all these cases, the wine growing center with a lower IAOe average is located at a higher and cooler altitude, which indicates larger possibilities for its adaptation to climate change. Assessed according to the latitudinal distribution the IAOe variability is found to diminish from the northern half, where all types of wine production can be obtained, to the southern half where suitability for QRW predominates.

One of the most important results of our study is the revealing of the changes in temperature, precipitation and sunshine duration for the wine growing centers from Romania. IAOe increases is caused by the rising temperatures and insolation, against the relative stability of precipitation. The results of our study regarding the distribution of temperature increases, sunshine duration and precipitation diminishing are consistent with those characterizing the particularities of climate change across the Romanian territory (Dumitrescu et al., 2014; Piticari and Ristoiu, 2012; Sfâcă, 2015).

Our study demonstrates once again that wine types production is determined by well defined climate suitability ranges specific to restraint areas, as is the case of the analysed wine growing centers. These ranges of climate suitability remained quite stable over time and allowed the establishing of the representative wine grape assortments and wine types for the vineyards. Changing the boundaries of these climate suitability ranges as an effect of climate change involves changing the types of wine production and wine grape assortments of vineyards. Today, the cool climate vineyards within traditional areas of vine culture have larger possibilities of adaptation to climate change, mainly to rising temperatures. On the other hand, as earlier research revealed (Jones et al., 2005) wine regions in warmer climate, in Romania's case southern wine regions (Dwr, DTwr and STwr), will face exceedings of climate suitability ranges for quality wine production. This will force them either to change their specific type of wine production by replacing their traditional wine grape variety assortments or to implement adaptation measures to climate change (Keller, 2010).

This study offers a scientifically based support to assess the impact of climate change on Romanian wine growing centers climate and to outline strategies of their adaptation to this phenomenon. Obtaining accurate depictions than those provided by this climate based study requires fine-scale analysis also of vineyards topography and soils, which can be achieved by using methodologies adapted to temperate continental climate characterizing Romania (Irimia et al., 2013, 2014).

Conclusions

By using the daily averages of temperature, precipitation and sunshine duration for the 1961 to 2013 time period at a 0.1 degrees resolution (10×10 km), our study provides averages for the 1961 to 1990 and for the 1991 to 2013 of the oenoclimate aptitude index IAOe for Romanian wine regions. The comparative study of the averages and the spatial distribution of the IAOe between the two time periods highlights major changes

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in climatic suitability for wine production in the last five decades in all wine growing centers of Romania. Except for the wine centres from Transylvania (Twr), in all wine growing centers the average of the IAOe moved during the 1991 to 2013 to the upper suitability class as compared to 1961-1990. Current IAOe averages reveal an improvement of climate suitability for the wine production in wine growing centers of Romania and the occurrence of climatic conditions to diversify the types of wine production. The study highlighted the negative evolution of climate suitability for wine production within the wine growing centers from the extreme south of Romania, where the current climate becomes more and more suitable for table grapes production. Further studies on the development of chemical composition and on the sensory profile of grapes and wines produced in this wine centers will clear up the way of these developments in the coming years.

The changes in climate suitability revealed by this study are likely to generate changes in wine grape assortments and traditional types of wine production of Romanian wine growing regions. Changes in wine grape assortments argued as adaptation to climate change are happening nowadays in Romania viticulture areas, but they are not integrated in a national strategy based on scientific support, but more on intuition of local wine producers and on their wish to diversify the wine types production. Our study provides the necessary support to outline such a national strategy and also to guide policy makers and wine growers in their work on adaptation of viticulture to climate change.

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ALLELOPATHIC EFFECT OF *SILYBUM MARIANUM* L. GAERTN. ON GROWTH AND NUTRIENT UPTAKE OF WINTER WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract. *Silybum marianum* (L.) Gaertn. is a widely known species, however, little is known about its allelopathic potential. *S. marianum* is a common weed in some crop areas. In this research, the allelopathic effect of *S. marianum* extracts on *Triticum aestivum* L. was investigated. The water extracts were used to show its phytotoxicity potential. The examined concentrations (m/v%) were: 1%; 2%; 3%; 4% and 5%. The control was tap water. The fresh and dry weight, the length of coleoptile and the radicle, the element contents of winter wheat seedlings were measured. The low concentration (1%) *S. marianum* extract had positive effects on the measured parameters. The fresh weight, dry weight and length of the radicle and shoot decreased at higher concentrations. The fresh weight of shoot decreased with 13% at the 5% treatment. The fresh weight of the radicle decreased with 32%, 42% and 67% at the 3%, 4% and 5% water soluble extract treatments. The decrease was significant. The higher concentrations had negative effects on the fresh weight of the shoot and especially of the radicle. The concentration of Ca, Mn, Mo and P increased in the radicle when winter wheat was treated with *S. marianum* extract, while the Fe and Zn concentrations did not change significantly. Increasing was determined in the N, K and Mg concentration both in the shoot and radicle of winter wheat. According to the results, one possible way is to use of milk thistle active ingredients as natural herbicides in the future. Our results show that we have to pay attention on milk thistle when it is appeared on field such as weed, because it has strong allelopathic effects.
Keywords: *biomass production, milk thistle, nutrient uptake, plant growth inhibition*

Introduction

The basic approach used in allelopathic research for agricultural crops has been to screen both crop plants and natural vegetation for their capacity to suppress weeds (Alam et al., 2001).

The word allelopathy is derived from two separate words. Allelon means “of each other”, and pathos means “to suffer”. Allelopathy refers to the chemical inhibition of one species by another. The “inhibitory” chemical is released into the environment where it affects the development and growth of neighbouring plants.

Allelopathic chemicals can be present in any part of the plant. They can be found in the leaves, flowers, roots, fruits or stems (Cheng and Cheng, 2015). They can also be found in the surrounding soil. Target species are affected by these toxins in many

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different ways. The toxic chemicals may inhibit shoot/root growth, they may inhibit nutrient uptake, or they may attack a naturally occurring symbiotic relationship thereby destroying the plant's usable source of a nutrient.

The effects of allelopathic toxins on sensitive plants can easily be tested in the lab or greenhouse setting. Seeds are the easiest and least expensive to test. Seeds that do not germinate in the presence of allelotoxins are probably displaying toxicity effects. Plants that become chlorotic and eventually die in the presence of allelotoxins are also showing signs of toxicity to the chemical. The allelopathic interactions include weed-weed, weed-crop and crop-crop are investigated in wide range (Elhaak et al., 2014; Lehoczky et al., 2011; Lehoczky and Gólya, 2013;).

Silybum marianum L. Gaertn. is a member of the Asteraceae family. The genus *Silybum* contains two species *S. marianum* (L.) Gaertn. and *S. eburneum* Coss. and Durieu (Adzet et al., 1993; Penksza and Szerdahelyi, 2009).

Each stem ends in a flower head about 5 cm in diameter (Montemurro et al., 2007), of red-purple colour. *S. marianum* is a long-day plant. In Israel, *S. marianum* occurs in two types, one type has purple flowers, while the other has white flowers (Vaknin et al., 2008).

The weight of 1000 *S. marianum* seeds is 28-30 g (Anderzejewska et al., 2011). Each flower head produces about 190 seeds, with an average of 6350 seeds per plant (Dodd, 1989). In the soil, seed can remain viable for up to nine years (Sindel, 1991). The seeds show little or no dormancy.

Silymarin is a lipophilic extract from the seeds of *S. marianum* and is composed of three isomer flavonolignans, silybin, silydianin and silychristin. Silymarin was first isolated in 1968 (Wagner et al., 1968).

Silybin is the component with greatest degree of biological activity and comprises 50-70% of silymarin. Seeds of *S. marianum* contain small amounts of flavonoids and approximately 20-35% fatty acids and other polyphenolic compounds (Ramasamy and Agarwal, 2008). A number of other flavonolignans have also been found in the seeds including isosilybin, dehydrosilybin, desoxysilycristin, desoxysilydianin, silandrin, silybinome, silyhermin and neosilyhermin (Kvasnicka et al., 2003).

Silybum marianum is native to the Mediterranean region of Europe and in Africa. *S. marianum* occurs especially in the wheat fields in Egypt and in Tunisia (Lehoczky et al., 2014).

Blessed milk thistle (*Silybum marianum* L. Gaertn.) is cultivated as a medicinal plant on 1500-2000 hectares, in Hungary (Borbélyné Hunyadi, 2010). It is important to know about its allelopathic effect againts the other plants (cultivated plants or a weeds). The allelopathic effects of *S. marianum* is important in crop production practice, because the residues could be incorporated into the soil after harvest. In countries, where it appears as a weed, this allelophatic effect may influence the result of the crop-weed competition as well.

The present study was conducted to evaluate and determine the initial growth performance and nutrient uptake of winter wheat under different extracts concentration of *S. marianum*.

Materials and methods

The *Silybum marianum* (L.) Gaertn. leaf samples were dried on 45 ºC. After drying, the sample was chopped than milled with grinder. The powdered sample was kept in paper bags until further measurements. The plant material of *S. marianum* was collected on an arable area.

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100 ml of the 10% concentration of water extract was made by soaking 10 g plant sample powder in 100 ml tap water. The mixture was kept at room temperature for 24 hours and was mixed occasionally. After that, the extract was filtered through MN 619 G ¼ filter paper. The following water soluble extract concentrations (m/v%) were used during the experiment: 5%, 4%, 3%, 2% and 1%. Tap water was used as a control (0%).

Before the experiments, the winter wheat seeds (*Triticum aestivum* L.) were sterilized with hydrogen peroxide. After the sterilization, the seeds were dried on paper towels. Twenty-five seeds of winter wheat were placed separately into 18 cm diameter Petri dishes on MN 640 m filter paper. The number of replications was four. 15 ml of each concentration of extracts were added to the Petri dishes. All of them were placed in a Binder APT Line KBW plant growth chamber at 21±1 °C for 7 days.

Seven days after the start of the experiments, the shoots and roots of every seedling were measured with millimetre paper. One hundred repetitions were performed. The fresh weight of the shoots and radicles was measured at the same time. The air dry weight was also measured. The shoot/root ratio was calculated using the dry weight.

The substances with allelopathic effects of the *S. marianum* sample have been investigated by HPLC. According to the methanol extract, 1 g plant sample contained 63 µg rutin and 194 µg salicylic acid. According to scientific papers, both rutin and salicylic acid have inhibitory effects on germination (Basile et al., 2000; Szepesi, 2009).

The element concentrations were measured by ICP-OES. The number of laboratory readings for ICP was the mean of two samples. The nitrogen concentration of samples was done with the Kjeldahl method. ANOVA (Analysis of Variance) was used to value the significant differences. On the figures the error bars indicate the standard deviation and the a–e indicate significant differences within treatments at the 5% level of probability according to Duncan's test. For the statistical analysis Sigma Plot 12.0 and MSTAT were used.

Examinations were carried out in three biological replications at different times with four replications. The results are the averages of these data. The experiments were conducted in controlled environmental conditions, so there was no difference between the results of three biological replications. Data of three repetitions were summarized and used for statistical analysis – results were calculated from twelve data points.

Results

Usually, the effects of allelopathy include reduced seed germination and seedling growth. First of all, the effect of *S. marianum* was observed at the fresh weight of shoot (coleoptile) and radicle (root) of winter wheat (*Fig. 1, Fig. 2*).

The positive effect was observed when 1% and 2% water-soluble extract treatments were applied. The fresh weight of shoot is significantly increased with 27% at the 1% treatments. This increasing was 25% at the 2% extract treatment. The higher concentrations had negative effect on fresh weight of shoot and especially of radicle. The fresh weight of shoot decreased with 13% at the 5% treatment. The fresh weight of the radicle significantly decreased with 32%, 42% and 67% at the 3%, 4% and 5% water soluble extract treatments.

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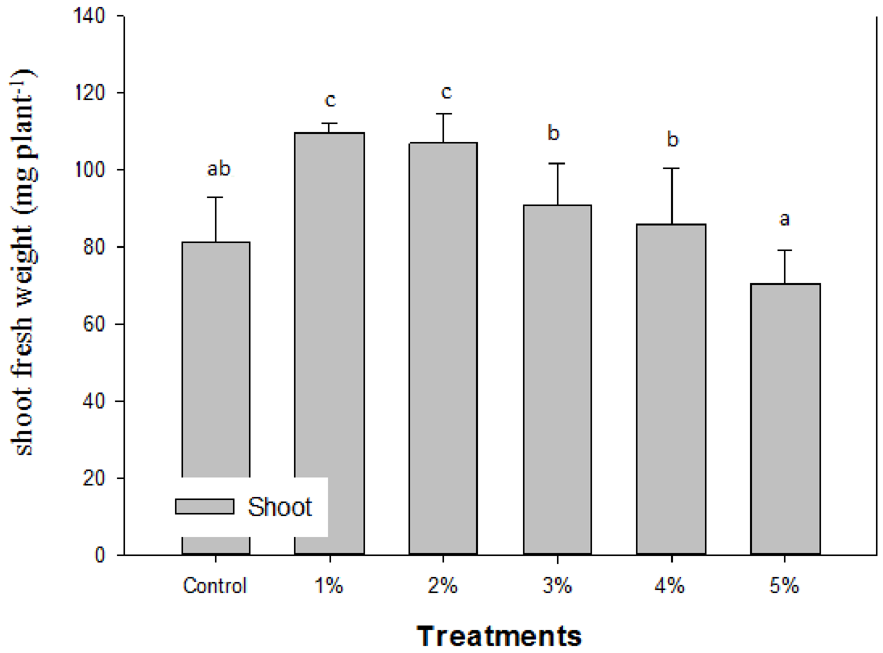

Figure 1. *The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the fresh weight of shoot of winter wheat (LSD$_{5\%}$ 20.3) n=12± S.D.*

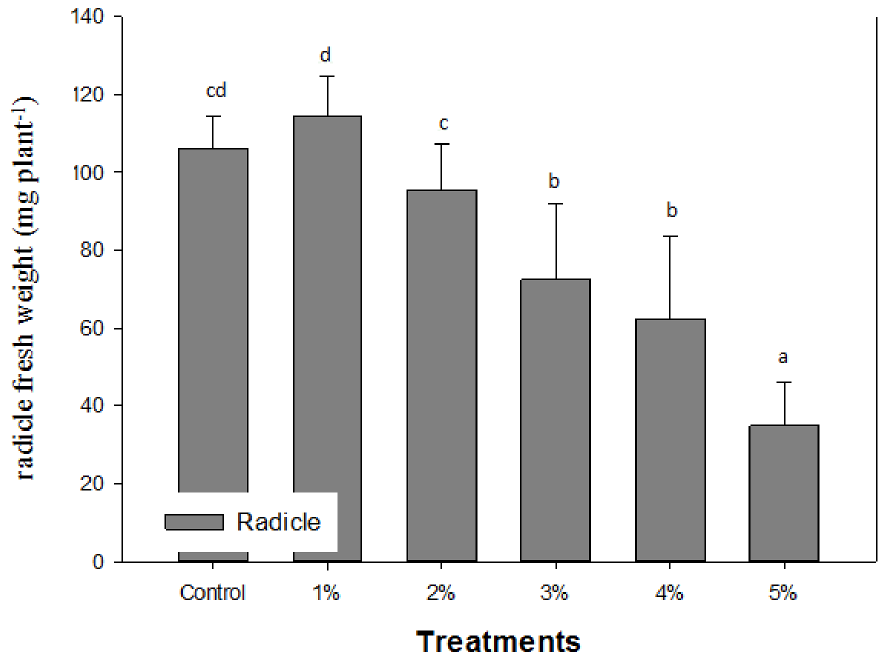

Figure 2. *The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the fresh weight of root of winter wheat (LSD$_{5\%}$ 19.24) n=12± S.D.*

At the end of the experiment, the dry weight of shoots and radicle also were measured (*Fig. 3, Fig. 4*).

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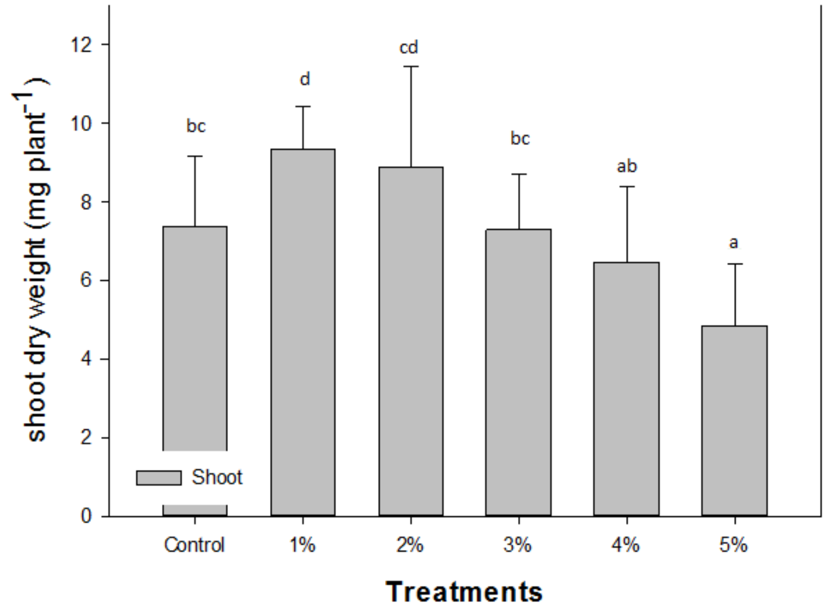

Figure 3. *The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the dry weight of shoot of winter wheat ($LSD_{5\%}$ 21.93) n=12± S.D.*

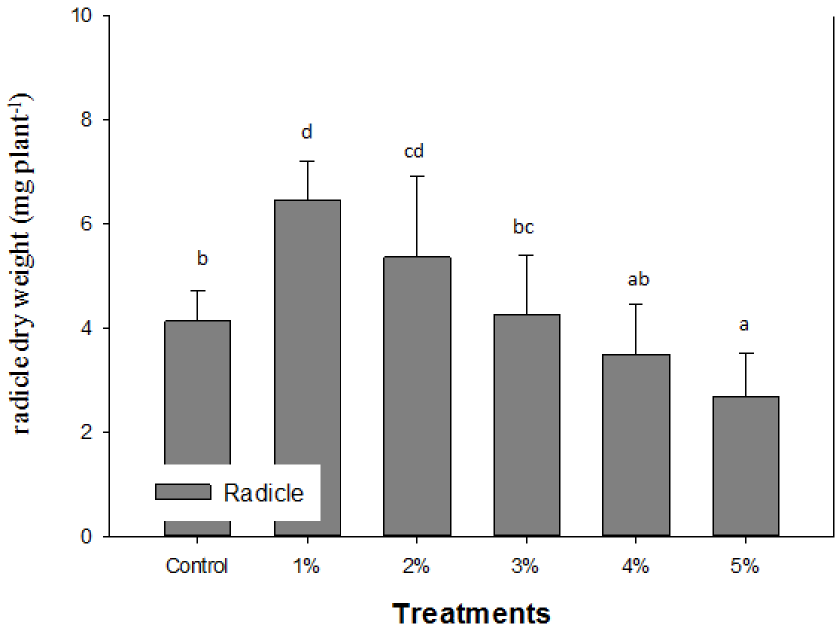

Figure 4. *The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the dry weight of root of winter wheat ($LSD_{5\%}$ 10.05) n=12± S.D.*

The 1% extract treatment had a positive effect on the dry weight of the shoot. The dry weight increased by 21%. The decrease seen at the higher extract concentration (3-5%) had a negative effect on the dry weight of the shoot. This decrease is parallel to an increase in extract concentration. The dry weight of the radicle was significantly increased when the seeds were treated with 1% extract. There was an increase of 36%

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compared to the control. Also, this increase was observed at the 2% extract treatments and when 3% extract were examined the results were around the control level. The dry weight of the radicle decreased at the 4% and 5% extract treatments. The decrease was higher at the 5% extract treatment.

Fig. 5 shows the different extract treatments on the shoot/radicle dry weight rate of winter wheat. The greater degree of decrease of radicle weight was expressed also in the shoot/radicle dry weight rate, which was significantly higher in case of 4% and 5% extract treatments compared to the control.

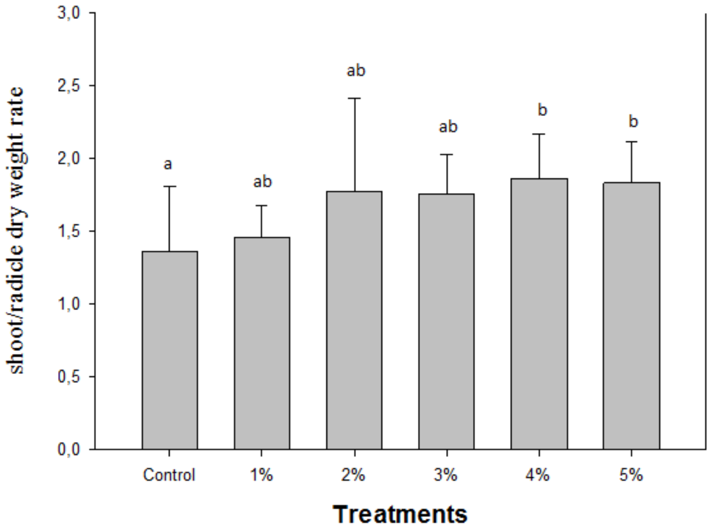

***Figure 5.** The effect of 1; 5; 7.5 and 10% Silybum marianum (L.) Gaertn. extracts on the shoot/root dry weight rate of winter wheat ($LSD_{5\%}$ 0.21) n=12± S.D.*

The length of shoot increased with 31.5% at the 1%, and increased with 23% at the 2% extract treatment (*Fig. 6*). The length of shoot decreased with 16% at the 4% and 40% at the 5% *S. marianum* extract treatments.

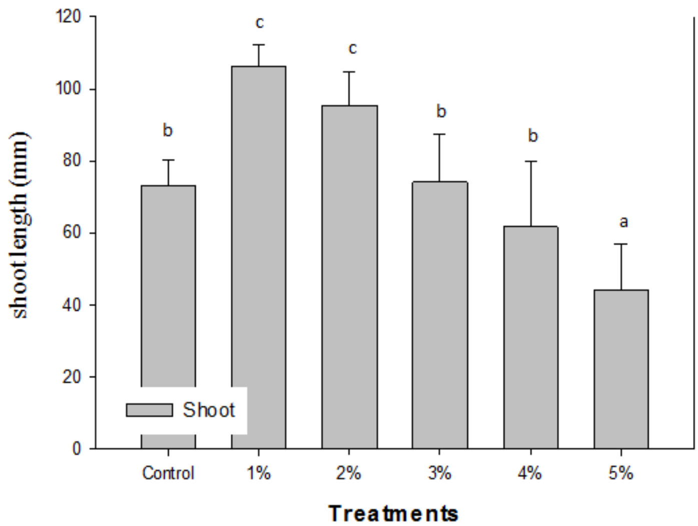

***Figure 6.** The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the length of shoot of winter wheat ($LSD_{5\%}$ 9.82) n=300± S.D.*

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The length of radicle decreased with 18% at the 3% with 30% at the 4% and with 58% at the 5% water soluble *S. marianum* extract treatments (*Fig. 7*).

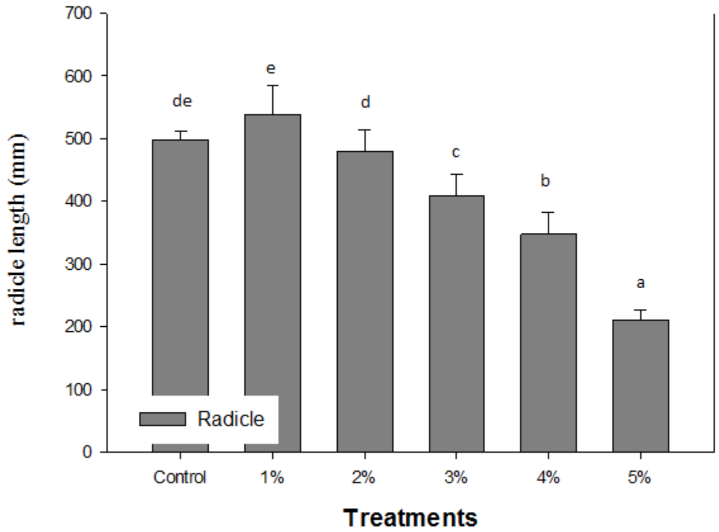

Figure 7. *The effect of 1; 2; 3; 4 and 5% Silybum marianum (L.) Gaertn. extracts on the length of root of winter wheat ($LSD_{5\%}$ 7.52) n=300± S.D.*

The elements concentrations of shoot and radicle also were measured at the end of the experiment (*Tables 1-2*).

Table 1. *Element concentrations of shoot of winter wheat treated with 1, 2, 3, 4 and 5% S. marianum extract treatments (mg kg-1, except N (%)) n=2± S.D.*

	N	**P**	**K**	**Ca**	**Mg**
Control	4.5±0.3 a	7714±593 a	14302±2258 a	1296±12 a	1447±98 a
1%	4.6±0.1 a	7327±521 a	26781±1122 ab	2741±306 c	1513±40 ab
2%	4.7±0.1 a	6854±314 a	38548±1240 bc	2256±95 b	1561±93 ab
3%	4.7±0.1 a	6967±588 a	42869±1879 bc	1562±47 a	1607±34 ab
4%	5.1±0.0 b	8073±889 a	55155±1672 c	1291±111 a	1766±252 b
5%	5.5±0.1 c	7180±901 a	41012±3403 bc	1211±16 a	1646±216 ab
Mean	*4.8*	*7353*	*36444*	*1726*	*1590*
$LSD_{5\%}$	*0.4*	*1620*	*19743*	*373*	*258*
	Fe	**Mn**	**Cu**	**Zn**	**Mo**
Control	62.6±3.5 a	33.3±5.3 a	9.6±0.9 c	42.4±2.9 a	0.51±0.15 a
1%	67.8±4.0 ab	33.4±0.8 a	6.1±0.2 ab	45.1±4.9 a	0.52±0.02 a
2%	69.6±1.0 ab	32.9±0.3 a	5.8±0.3 ab	46.7±0.1 a	0.52±0.05 a
3%	74.6±7.3 b	32.5±2.3 a	5.3±0.5 a	44.8±2.5 a	0.53±0.03 a
4%	64.8±3.7 ab	28.4±2.1 a	5.8±0.2 ab	48.4±1.3 a	0.47±0.09 a
5%	61.2±4.9 a	30.6±0.1 a	7.1±0.8 b	47.4±5.4 a	0.51±0.00 a
Mean	*66.8*	*31.9*	*6.6*	*45.8*	*0.51*
$LSD_{5\%}$	*12.0*	*7.3*	*1.2*	*8.5*	*0.21*

Note: different letters (a,b,c) indicate significant difference, values denoted by the same letter are not significantly different at the p=0.05 level of probability (Duncan's Multiple Range Test).

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The concentration of manganese, zinc, molybdenum and phosphorus in the shoot did not changed significantly at the treatments compared to the control and to each other.

Table 2. *Element concentrations of radicle of winter wheat treated with 1, 2, 3, 4 and 5% S. marianum extract treatments (mg kg^{-1}, except N (%)) n=2± S.D.*

	N	**P**	**K**	**Ca**	**Mg**
Control	3.3±0.3 ab	3451±180 a	11355±984 a	1863±381 a	1981±208 a
1%	3.3±0.4 ab	3285±308 a	14222±1506 a	4884±225 b	3757±166 b
2%	3.1±0.1 a	4395±733 ab	35925±1326 b	5968±178 c	5720±70 d
3%	3.6±0.0 b	4658±669 b	60643±1534 c	5757±233 c	5017±302 c
4%	4.3±0.1 c	4746±193 b	69972±1288 d	6054±229 c	4723±183 c
5%	5.0±0.1 d	5200±435 b	70208±1586 d	6839±249 d	4994±387 c
Mean	*3.7*	*4289*	*43721*	*5228*	*4365*
$LSD_{5\%}$	*0.5*	*1116*	*3769*	*712*	*534*

	Fe	**Mn**	**Cu**	**Zn**	**Mo**
Control	70.6±2.6 a	31.6±3.8 a	29.8±3.4 b	55.4±5.6 a	0.34±0.03 a
1%	66.5±3.6 a	33.8±3.8 a	11.8±1.7 a	45.6±14.8 a	0.36±0.00 ab
2%	71.5±7.7 a	40.9±2.7 ab	13.5±1.4 a	49.0±13.4 a	0.32±0.04 a
3%	61.4±4.2 a	40.4±2.6 ab	10.7±1.5 a	45.9±2.3 a	0.44±0.02 cd
4%	64.9±5.8 a	44.0±6.0 b	11.2±3.2 a	42.6±1.6 a	0.40±0.00 bc
5%	67.8±5.2 a	48.2±3.6 b	22.8±3.2 b	55.2±8.3 a	0.47±0.01 d
Mean	*67.1*	*39.8*	*16.6*	*48.9*	*0.39*
$LSD_{5\%}$	*12.5*	*8.5*	*7.1*	*16.3*	*0.05*

Note: different letters (a,b,c) indicate significant difference, values denoted by the same letter are not significantly different at the p=0.05 level of probability (Duncan's Multiple Range Test).

The concentration of potassium, magnesium and calcium increased in the radicle of winter wheat at all treatments. The highest increasing could be observed at the 5% water soluble extract treatment. Increasing was determined in the nitrogen, magnesium and potassium concentration both in the shoot and radicle of winter wheat.

The concentration of phosphorous increased in the radicle when wheat was treated with *S. marianum* extract. Significant increasing was observed at the 3%, 4% and 5% extract treatments in the radicle compared to the control.

Discussion

Our results show that we have to pay attention on milk thistle when it is appeared on field such as weed, because it has very strong allelopathic effects.

S. marianum is a major weed in sugar beet, winter wheat and canola causing large yield reductions (Khan and Marwat, 2006; Shimi et al., 2006). However, researchers have documented the allelopathic effect of *S. marianum* on mustard, cucumber, wheat and sorghum (Inam and Hussain, 1988).

The allelopathic potential of *S. marianum* increased with increased concentration. In addition, stability and level of phytotoxicity of *S. marianum* vary on methods for extract preparation and solvent or media. The fresh water extract is more toxic than the heated

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extract, but in both condition there is some inhibitory substances presence in *S. marianum* tissues causing this allelopathy.

The aqueous extract of leaves of *S. marianum* were assayed at different concentration (1, 2, 3, 4 and 5%) to assess their allelopathic potential. The extracts were tested on seeds of winter wheat (*T. aestivum*). The seedling shoot and radicle lengths were significantly reduced with 40% (shoot) and 58% (radicle) by 5% extract compared to the control. Organic extracts have reduced the seedlings growth in high percentage at the 5% water soluble extract treatments. Our finding also reporting that the radicle growth of winter wheat is suppressed more than its shoot growth, since the fresh weight of shoot decreased with 13%, while radicle with 67% at the 5% treatment.

The phosphorus concentration in the radicle is increased when wheat was treated with milk thistle waterextract. The P is requirement for optimal growth. The probability of P toxicity increases at concentrations higher than 10 mg g^{-1} DW. P toxicity in plants is rare, because plants down-regulate their P transporters involved in net P uptake from the root environment when supplied with more P than required for optimum growth (Dong et al., 1999).

Over all the allelopathic potential of *S. marianum* increased with the increasing concentration.

Weeds are an important variable to consider in crop production (Bilalis et al., 2010; Lehoczky et al., 2015). It is the opinion of the research committee that weed science will be advantageously positioned for the future if research focuses on research-decision processes, weed biology and ecology, weed control and management practices, herbicide resistance, issues related to transgenic plants, environmental issues, and potential benefits of weeds (Hall et al., 2000).

According to our results water extract of milk thistle has very strong allelopathic effect, so special attention needs when this plant appears as weed.

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DETERMINATION OF HETEROSIS AND HETEROBELTIOSIS VALUES OF SALT-TOLERANT SUMMER SQUASH (*Cucurbita pepo* L.) GENOTYPES AND GENETIC RELATIONSHIPS OF PARENTAL GENOMES

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Abstract. This study was carried out to determine the heterosis and heterobeltiosis values in F1 hybrids which are obtained through hybridization of salt-tolerant genotypes in summer squash (*Cucurbita pepo* L.). The materials used were the promising hybrid combinations and their parents, which are previously reported among the salt-tolerant genotypes by detecting the influence of parents on heterosis and heterobeltiosis values in advanced hybridization studies. Eight domestic summer squash genotypes (Tutluca 1, Tutluca 2, Halfeti 1, Halfeti 2, Bozova Mrkz, Yaslıca, Konak and Şanlıurfa Mrkz), were chosen from salt-tolerant genotypes and 28 F1 hybrids obtained through hybridization of these genotypes. The heterotic influences were remarkably important, and it was possible to get promising hybrids in terms of examined traits. Parents were evaluated for plant length (cm), stem diameter (mm), plant width-spread (cm), number of leaves (number), 50% female flowering time (day), yield (kg), parental and hybrid heterosis and heterobeltiosis values (%), and parental contribution to heterosis and heterobeltiosis of F1 hybrids. The highest heterotic effect was determined in the summer squash yield traits. In the yield, a maximum of 32.67% heterosis and 24.36% heterobeltiosis values were obtained. Additionally, genetic relationships of parental genotypes based on chloroplast DNA sequence analysis were compatible with results of heterosis and heterobeltiosis.
Keywords: *Cucurbita pepo* L.*, abiotic stress, breeding, hybridization, trnL-F, genetic similarities*

Introduction

Among all agricultural products, vegetable production in the world reached its highest rate (45%) between 1985 and 1995 (Harrison, 2002). Turkey's fruit and vegetable products have an important role in both industry and in exports. Agricultural products constitute approximately 25% of Turkey's exports. However, vegetables share in this value is only around 2% (Turkstat, 2015). Eating habits are changing in favor of vegetables parallel to cultural and economic developments in Turkey, as in many other countries throughout the world. Fruits and vegetable crops have a remarkable place in the diet of developed countries (Skreden et al., 2017). Summer squash (*Cucurbita pepo* L.) which has an annual production of 137,000 Hg/Ha (Anonymous, 2013), is produced in a wide number of climate zones, has significant economic value (Paris, 1986). Summer squash has a wide variation in terms of size, color and shape of the fruit (Paris, 1986). *C. pepo* is divided into eight groups in terms of fruit shape, six groups in terms of fresh consumption of the fruit and five groups based on the thickness of the fruit skin.

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The most distinctive feature of summer squash from other squash types is that fruits are almost always smooth (Paris, 2008).

Plant breeders' ultimate target is high production/yield. To widen genetic variation for breeding, variety expansion and hybridization are carried out. The most important criteria for hybridization is to select the most suitable parents for this purpose in early generations. For a breeder trying to develop a variety, finding heterosis values means the work is well-directed, and may produce a variety in a shorter time period. A successful breeding programme is based on accurate selection of the material used. Determination of heterosis values is important to develop new and strong squash cultivars (Stuber, 1994). Hallauer and Miranda (1988) calculated heterosis values according to potential gene combinations of parents. The best combinations were achieved from the hybridization between the types and lines in different heterotic groups. The width of the heterotic pattern in genetic sources affected the degree of heterosis level positively.

Heterosis, defined as the superiority of F1 over its better parent, will occur when F1 > P1, if it is the better parent for the property under consideration, or F1 < P2 if P2 is the better parent. Clearly, P1 will be the better parent where superiority is equitable to a higher value for the mean phenotype, e.g., higher yield, whereas P2 will be the better parent where it is equitable to a lower value for the mean phenotype, e.g., fewer days to reach maturity. Heterosis of the first kind, which called positive, will occur when;

$$F1 - P1 = [F11/2\,(P1 + P2)] - [1/2\,(P1 - P2)] \qquad \text{(Eq. 1)}$$

Heterosis of the second kind, which is called negative, will occur when;

$$F1 - P2 = [F1 - 1/2\,(P1 + P2)] + [1/2\,(P1 - P2)] \qquad \text{(Eq. 2)}$$

The heterosis effect in summer squash is seen primarily in the number of fruits per plant and in earliness of fruit development (Curtis, 1939). Curtis (1941) reported heterosis in the earliness of fruit harvest 114% over the parental mean and 87% over the better parents mean. A comparison of nine yellow fruited F1 hybrids with three commercial varieties showed 46% advantage in earliness and 19% in yield (Elmstrom, 1978).

The F_1 must be superior to its parents to observe heterosis, whether it is also superior to all of the pure-breeding lines. Heterosis is theoretically a quadratic function of parental genetic distance (GD) at the underlying quantitative trait loci (QTL) for the trait considered (Falconer and Mackay, 1996; Melchinger, 1999). Experiments with maize showed an increase in heterosis with increasing parental GD (Moll et al., 1962; Melchinger, 1999), but they also suggested an optimum level of parental GD, after which heterosis and hybrid performance declines (Moll et al., 1965). The results provide strong evidence that epistasis plays a major role as the genetic basis of heterosis (Yu et al., 1997). Programs to exploit mating systems to produce hybrids were developed in spinach (monoecious forms), cucumber (genetically controlled), squash (response to chemicals), and carrot (cytoplasmic male sterility) (Pearson, 1983).

Chloroplast DNA has been used extensively to determine the genetic similarities and differences in plants. The chloroplast *trn*L-F region has proven to be phylogenetically useful from the species level to the family level. It is a noncoding region that includes the trnL (UAA) intron ranging from 350 to 600 base pairs (bp) and the intergenic spacer

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between trnL (UAA) 3′ exon and the trnF (GAA) gene. That sequence is in the region where the chloroplast genome does not code. It is widely used in phylogenetic research (Taberlet et al., 1991). Additionally, it comes to forefront in molecular similarity studies due to its characteristics, such as the structural stability of chloroplast DNA, haploidy (n), uniparental transferability (in general) and non-recombinant nature (Small et al., 2004). In molecular systematic studies, a phylogenetic tree is built with the data obtained while showing the degree of relationships among genotypes (Saitou and Ġmanishi, 1989).

Determination of heterosis and heterobeltiosis rates in F1 hybrids through hybridization of salt-tolerant genotypes and with DNA based relationships of parental genotypes were the main aims of this study. Since salinity is known to be a great handicap for summer squash cultivation, salt-tolerant genotypes (Tutluca 1, Tutluca 2, Halfeti 1, Halfeti 2, Bozova Mrkz, Yaslıca, Konak and Şanlıurfa Mrkz) were used to evaluate the effect of parents on heterosis and heterobeltiosis.

Materials and Methods

Material

Eight different summer squash pure lines (*C. pepo* L.) were used in this study (*Table 1*). The parents were purified between 2008 and 2011. In addition to studies on their yield and yield components, a study of their tolerance to salt stress was published in 2012 (Karipcin and Sari, 2012). The summer squash parental lines used as plant material (*Table 1*) and hybridizations scheme without reciprocals (*Table 2*) are given below.

***Table 1**. Parental Summer squash Genotypes used in the research (C. pepo L.)*

Parent name	Origin
Tutluca1	Tutluca-Bozova
Tutluca2	Tutluca-Bozova
Halfeti1	Halfeti-Şanlıurfa
Halfeti2	Şanlıurfa
Bozova Mrkz.	Bozova
Yaslıca	Yaslıca-Bozova
Konak	Konak-Şanlıurfa
Şanlıurfa Mrkz.	Şanlıurfa

***Table 2**. Hybridization scheme used in the research*

Hybridization without reciprocal		Parents							
		Tutluca 1	**Tutluca 2**	**Halfeti 1**	**Halfeti 2**	**Bozova Mrkz.**	**Yaslıca**	**Konak**	**Şanlıurfa Mrkz.**
Parents	Tutluca1	-	X	x	x	x	x	x	x
	Tutluca2	-	-	x	x	x	x	x	x
	Halfeti1	-	-	-	x	x	x	x	x
	Halfeti2	-	-	-	-	x	x	x	x
	Bozova Mrkz.	-	-	-	-	-	x	x	x

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	Yaslıca	-	-	-	-	-	-	x	x
	Konak	-	-	-	-	-	-	-	x
	Şanlıurfa Mrkz.	-	-	-	-	-	-	-	-
Total Hybrids		$28F_1$ Hybrids							

Definition of Research Area

Harran Plain is located between 36^{o} 47^{1} and 39^{0} 15^{1} east longitudes and 36^{0} 40^{1} and 37^{0} 41^{1} north latitudes in Şanlıurfa province in the Southeastern Anatolia, Turkey. The area is surrounded by Urfa Mountains in the North, Turkey-Syria border in the South, Tektek Mountains in the East and Fatik Mountains in the West. Harran Plain covers 225.109 ha (Anonymous, 1980) and is 410 m above sea level.

General Soil Characteristics of the Research Area

The research was carried out in Ikizce region. Thesoil in this region has a structure whose topography is flat or nearly flat; the whole profile is limy with a clay texture. Soil pH varies between 7.3-7.4, with 1.1% organic material on the surface and 0.8% in the deep soil zones. In the soils evaluated, field capacity in dry weight, the wilting point and bulk density ranges were between 32.71-33.84%; 21.18-22.55 and 1.37-1.41, respectively (Dinc et al., 1988; Almaca and Gok, 1997).

General Climate Characteristics of Research Area

Although Sanlıurfa is in the South-Eastern Anatolia climate zone, it is under the influence of the Mediterranean climate. It has hot and dry summers, and warm winters. The precipitation increases from south to north and from west to east. According to multi-year averages, July is the warmest month (46.8^{0}C), while maximum precipitation (93.1 mm) and the highest moisture (71%) occurs in January (Anonymous, 2012).

Method

A total of 28 hybrids were obtained from the hybridization of eight parents in 2013. Thirty six summer squash genotypes (eight parents and 28 hybrids) were evaluated under field conditions according to randomized complete block design with three replicates in 2014. The research was conducted in the GAP Agricultural Research Institute fields in Koruklu Research Station. The plants were sown in a row with 1.5 m x 0.5 m spaces with ten plants in each parcel. The first and last plants in each row was used as buffer. In every parcel, plant length (cm), stem diameter (cm), plant width-spread (cm), number of leaves (number), 50% female flowering time (day), stem length (cm) and yield (kg) were analyzed.

Heterosis and Heterobeltosis Calculations

Heterosis level was calculated for each trait by subtracting parental average value from the F1 hybrid value and dividing this to parental average and multiplying by 100 (Fonseca and Paterson, 1968; Sari et al., 2003a; Virmani et al., 2003; Cho et al., 2004; Denizer et al., 2005; Jose et al., 2005; Ahmad et al., 2005); and by subtracting the

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superior parent's value from the F1 hybrid value, dividing it to superior parent's value and multiplying by 100 (Sari et al., 2003b; Cho et al., 2004; Denizer et al., 2005; Jose et al., 2005). That is, formulas to determine heterosis [(*F1-EO/EO*) *x 100*], and heterobeltiosis ratio [(*F1-ÜE/ÜE*) *x 100*], were used. The contribution of the parents to heterosis and heterobeltiosis was determined for evaluated traits. Differences between the genotypes were evaluated with JMP 5.1.2 software (SAS Institute, Cary, NC, USA).

DNA Isolation for Molecular Studies and PCR Conditions

DNA extraction was performed according to Karaca et al. (2005) by taking the leaf samples from each of eight parents used in this study. The quantitative features of the isolated DNA were measured with a spectrophotometer, and 1% agarose gel was used to measure its qualitative features. PCR reaction was optimized to reproduce the *trn*L-F region. In order to reproduce the *trn*L-F region, F: 5'ATTTGAACTGGTGACACGAG3', R: 5'GGTTCAAGTCCCTCTATCCC3' primer pairs were used. In the tubes with a reaction of 200 µL, "by taking 2.5 µL of reaction buffer with 10X", 2.5 µL from 25 mM MgCl, 0.4 µL from 10 mM dNTP, 1 µl from the primer pairs with 10 pmol/µL, 0.25 µL from Taq DNA polymerase, 2 µL from template DNA, 15.35 µL from dH2O, the total volume would be arranged to be 25 µL. To check for contamination during the PCR reaction, a negative control of non-genomic DNA was used. The PCR was adjusted to 35 cycles to remain at 94^0 C for five minutes, at 94^0 C for one minute, at 58^0 C for one minute and at 72^0 C for one-minute. The last PCR product was kept at 4^0 C. In order to see the region reproduced, 5 µL DNA from PCR product was mixed with 2 µL loading dye and loaded into 1.5% agarose gel. Later, with a gel scanning device, an image was taken under UV light. After appropriate band intervals were obtained, the sequence of the related region was done through service procedures.

In Silico Analysis of the Sequences

To confirm the accuracy of the sequences in the *trn*L-F regions of the eight parents taken in the study, BLAST analysis was carried out in the NCBI database. Sequences were then reset with Clustal X 2.0. To determine the genetic proximity and distance, these aligned sequences were subjected to MEGA 7.0 software to develop a genetic relationship tree. The Maximum Parsimony algorithm from the character-based methods was used and bootstrap, branch and bound analysis were done.

Results and Discussion

The average values of eight parents and 28 hybrids for analyzed traits, as well as heterosis and heterobeltiosis values of the F1 hybrids were given in *Appendix*. The parental contribution (%) to heterosis (*Table 3*), and heterobeltiosis (*Table 4*) were calculated. The error mean square values of these examined traits and a variation analysis are given in *Table 5*. Considerable differences for all traits were observed between the F1s and their parents. The average plant length of the parents and hybrids were measured as 29.04 cm and 29.57 cm, respectively (*Appendix*). The maximum heterosis value (9.60%) occurred in the 5 x 8 (Bozova Mrkz x Şanlurfa Mrkz) hybrids (*Appendix*), and the highest heterobeltiosis value

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(0.61 %) was observed in the 3 x 8 (Halfeti-1 x Şanlıurfa Mrkz.) hybrids for plant length (*Appendix*). An average of 3.29% heterosis in hybrids and -23.12% of heterobeltiosis shows the heterotic effects in the material used for the plant length. The parent with the highest contribution (6.52%) to the heterosis in plant length was Sanlıurfa Mrkz, followed by the Halfeti-1 genotype (4.07%). The lowest contribution for plant length was from Konak genotype (0.46%) (*Table 3*). The highest stem diameter was measured from Sanlıurfa Mrkz with 22.8 mm, and the lowest was 15.8 mm from Bozova Mrkz. The stem diameter average of the parents and the hybrids were found as 19.54 and 19.82 mm, respectively (*Appendix*). In terms of stem diameter, while the highest heterosis (9.13%) was obtained from the Halfeti-2 x Bozova (4 x 5) hybrid, and the highest heterobeltiosis value (5.08%) was from the Halfeti-1 x Şanlıurfa Mrkz (3x8) hybrid; the lowest heterosis (-4.51%) was seen in the Halfeti-1 x Yaslıca (3 x 6), and the lowest heterobeltiosis (-47.59%) was from Yaslıca x Şanlıurfa Mrkz (6 x 8) hybrids (*Appendix*). While Bozova Mrkz parent had the highest contribution to heterosis (5.61%) (*Table 3*) with a value of -6.31 %, the Halfeti-1 parent had the highest contribution to heterobeltiosis (*Table 4*). Plant width-spread was determined as 141.95 cm for parents and 146.31 cm for F1 hybrids, and the highest heterosis value (16.18 cm) was from the Tutluca-2 x Şanlıurfa Mrkz hybrid (*Appendix*). The lowest heterosis (8.03%) was obtained from Halfeti-1 x Halfeti-2 (3 x 4) hybrid combination (*Appendix*). According to heterobeltiosis values, the highest and lowest values were obtained as 5.62% from the Halfeti-1 x Şanlıurfa Mrkz (3 x 8) and -37.66% from the Tutluca-2 x Bozova Mrkz (2 x 5) hybrids (*Appendix*). When the parental contribution to heterosis and heterobeltiosis are analyzed for plant width-spread, it is understood that the Şanlıurfa Mrkz parent gives the highest contribution to heterosis and heterobeltiosis with (12.43%, -2.08%) (*Table 3* and *Table 4*). The average number of leaves for the parents was 47.21 pieces/plant, while for hybrids 50.03 pieces/plant; with a value of 19.13 pieces/plant Şanlıurfa Mrkz parent (number 8) having the highest heterosis value, and the lowest heterosis value (-7.05%), belonged to the Halfeti-1 x Yaslıca hybrid (*Appendix*). The highest contributions for number of leaves to heterosis and heterobeltiosis were 10.44 % and -8.28 from the Şanlıurfa Mrkz, and from Yaslıca parents with a 2.71% heterosis value, and Bozova Mrkz parent with a -31.81% heterobeltiosis value takes the last place in the order (*Table 3* and *Table 4*). According to 50% female flowering time, the parental average is 27.86 days; and hybrids average is 28.37 days (*Appendix*). The heterosis values for 50% female flowering time is given in *Appendix*. As reported in the *Appendix*, for 50% flowering time, the highest heterosis value (19.21%) belongs to the Tutluca-1 x Halfeti-1 hybrid (1x3) and the lowest heterosis value (-21.25%) belongs to the Halfeti-1 x Şanlıurfa Mrkz (3 x 8) hybrid. When the contribution of parents to heterosis (*Table 3*) and heterobeltiosis (*Table 4*) were examined for the 50% female flowering time trait, it was seen that the highest contribution to heterosis (5.28%) was from the Halfeti-2; the lowest contribution to heterosis (-4.9%) was from the Şanlıurfa Mrkz, and the highest contribution margin to heterobeltiosis (-10.63%) belongs to the Tutluca-2 parents. The lowest contribution to heterobeltiosis (-29.02%) was again from the Şanlıurfa Mrkz genotype. When stem length data were examined, the average of the hybrids (34.74 cm) was higher than that of the parents (32.43 cm). Among the parents the highest value (36.7 cm) belongs to the Bozova Mrkz. On the other hand, among

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hybrids, the highest average value (40.99 cm) was from the Tutluca-1 x Yaslıca (1 x 6) hybrid (*Appendix*). It was found that the heterosis value of the 4 x 8 hybrid (26.78 %) was the highest, and 3 x 4 hybrid was the lowest (-22.62%) (*Appendix*). In terms of the parental contribution to heterosis according to stem length, the Şanlıurfa Mrkz parents' value (19.64%) was the highest, Bozova Mrkz parents' value (-1.19%) was the lowest (*Table 3*). When yield value, which is one of the most important traits when developing a cultivar, is examined, it was concluded that the differences between parents and hybrids are much more distinctive. When parents and hybrids were examined for yield, the average was 1876.02 kg for the hybrids, and was 1636.25 kg for the parents. The highest yield for the parents belonged to the Sanlıurfa Mrkz. (2600 kg), and to a hybrid obtained from Konak x Şanlıurfa Mrkz (7x8) (3233.41 kg) (*Appendix*). When heterosis values were analyses for yield, the Bozova Mrkz x Şanlıurfa Mrkz (5x8) hybrid had the highest heterosis value (32,67 %), whereas the lowest heterosis (-13,17 %) was observed from the Halfeti-1 x Bozova Mrkz (3x5) hybrid (*Appendix*). The highest heterobeltiosis value (24.36%) was obtained from the Konak x Şanlıurfa Mrkz hybrid, and the lowest (-67.20%) was from the Tutluca-1 x Tutluca-2 hybrid (*Appendix*). When the contribution of the parents to heterosis and heterobeltiosis were examined, the highest contribution (27.15% and 0.18%, respectively) was obtained from Şanlıurfa Mrkz parent, and the lowest values were from Halfeti-1 (5.25%) and Tutluca-1 (-44.86%) parents (*Table 3* and *Table 5*).

For all the traits analyzed for heterosis in this study, the best results were observed from Şanlıurfa Mrkz genotype, except 50% flowering time, in which this parent was ranked last (*Table 3*). When the data was examined for the average of the hybrids, the Halfeti-1 x Şanlıurfa Mrkz (3x8) hybrid was recorded as having the highest values for plant length, stem diameter and plant width-spread traits, this hybrid had the lowest value for only 50% flowering time (*Appendix*). Tutluca-1 x Tutluca-2 (1x2) hybrid had the highest value for the leaf number (number/plant). While Tutluca-1 x Yaslıca (1x6) hybrid average was the highest for stem length (cm), and the Konak x Şanlıurfa Mrkz (7x8) hybrid had the highest yield average in their group. When the heterosis values were analyzed for all traits, it was concluded that heterotic influence was quite effective and for each trait, different hybrids presented their heterotic differences with varied values (*Appendix*, *Tables 3-4*).

***Table 3**. Contribution of parents to heterosis for the analyzed traits*

	Plant length (cm)	**Stem diameter (mm)**	**Plant width-spread (cm)**	**Number of leaves (number)**	**50% Female flowering time (day)**	**Stem length (cm)**	**Yield (Kg/Da)**
Tutluca1	1.78	2.63	3.01	6.53	2.17	8.24	8.15
Tutluca2	3.01	1.64	1.73	2.98	3.49	1.04	12.35
Halfeti1	4.07	3.14	2.23	6.24	3.13	3.31	5.25
Halfeti2	3.96	2.73	-1.11	5.42	5.28	7.48	9.36
Bozova Mr	2.56	5.61	2.61	4.09	0.54	-1.19	8.76
Yaslıca	3.98	2.24	-0.67	2.71	3.55	8.41	12.49
Konak	0.46	2.10	1.36	6.42	1.43	12.31	21.15
Şanlıurfa Mr	6.52	4.08	12.43	10.44	-4.90	19.64	27.15
Average	3.29	3.02	2.70	5.60	1.84	7.41	13.08

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***Table 4**. Contribution of parents to heterobeltiosis for the traits evaluated*

	Plant length (cm)	Stem diameter (mm)	Plant width-spread (cm)	Number of leaves (number)	50% Female flowering time (day)	Stem length (cm)	Yield (kg/da)
Tutluca1	-27.54	-13.07	-24.42	-27.81	-14.47	-4.99	-44.86
Tutluca2	-23.35	-14.67	-25.89	-31.53	-10.63	-8.48	-42.10
Halfeti1	-16.32	-6.31	-15.11	-25.60	-25.60	-12.37	-19.85
Halfeti2	-19.19	-8.16	-22.85	-29.15	-26.59	-9.41	-22.89
Bozova Mr	-34.91	-16.93	-26.40	-31.81	-22.72	-7.91	-44.34
Yaslıca	-32.54	-24.17	-25.03	-30.79	-28.31	-3.26	-41.42
Konak	-18.40	-11.48	-19.33	-20.26	-20.96	1.77	-7.48
Şanlıurfa Mr	-13.26	-9.73	-2.08	-8.28	-29.02	2.01	0.18
Average	-23.19	-13.07	-20.14	-25.66	-22.29	-5.33	-27.85

The hybrids range in a wide interval in terms of their heterosis values (*Appendix*). When the data was examined, the hybrids had a plant length average between -3.98 (1x7) and 9.6 (5x8), stem diameter average between -4.51 (3x6) and 9.13 (4x5), plant width-spread values between -8.03 (3x4) and 16.18 (2x8), leaf number averages between -7.05 (3x6) and 19.13 (7x8), 50% female flowering time averages between -21.25 (3x8) and 19.21 (1x3), stem length averages between -22.62 (3x4) and 26.78 cm (4x8), and yield average values between -13.17 (3x5) and 32.67 (5x8). These values were caused by the heterotic differences between the parents used in this study.

A high statistical significance of 1% among all the traits except stem diameter was obtained (*Appendix*). The error mean squares of the analyzed traits were significant and it highlights the existence of a wide variation among the materials tested (*Table 5*).

***Table 5**. Error mean square values of variance analyze and analyzed characteristics*

S.V.	Plant length (cm)	Stem diameter (mm)	Plant width-spread (cm)	Number of leaves (number)	50% Female flowering time (day)	Stem length (cm)	Yield (Kg/Da)
F	3.09**	2.46^{NS}	18.32**	11.18**	2.90**	59.07**	30.20**
Variety	79.04**	17.52	1478.67**	287.61**	91.50**	2769863**	1460006**

** 1% statistical significance exists.

When heterosis and heterobeltiosis values for yield traits were analyzed according to phylogenetic tree (*Fig. 2*), the Bozova Mrkz and Şanlıurfa Mrkz parents, which composed the Bozova Mrkz x Şanlıurfa Mrkz hybrid with the highest heterosis value are the members of two different groups; while the parents of the hybrid with the lowest heterosis value (Halfeti1 x Bozova Mrkz) belongs to same group of parents, and similarities between them are closer to each other. It was determined that the hybrids composed of the individuals that are phylogenetically farther from each other had higher yield values. Konak x Şanlıurfa Mrkz, which had the highest heterobeltiosis values, belonged to different groups, while the Tutluca1 x Tutluca2 hybrid had the lowest value, were the farthest members in the phylogenetic tree, but genetically, the fruits of

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both parents were small, and their contribution to the heterobeltiosis value was not sufficient.

When the literature was searched concerning our topic, it was seen that there are very few studies with regards to determining the heterotic characteristics in summer squash. Helmy (1993) identified the highest positive heterosis contribution for total fruit number and fruit weight per plant by evaluating 15 hybrids and parents in his study with six inbred agoor lines (*Cucumis melo var. chata*, L.) El-Adl et al. (1996) reported parallel results with our study by identifying a positive influence of heterosis contribution on yield. Pradip et al. (2013) conducted a study on the yield and antioxidant contents of 28 Luffa *acutangula* (Roxb.) L. genotypes (7 of them were parents), and reported that genetic variability was quite effective on heterosis and on general and specific combining abilities, similar to our study. Iathet and Piluek (2006) reported that the number of fruit and yield per plant in melons had high heterosis values (0.61 and 0.60, respectively) due to genetic differences; and they found high correlation between number of fruits and yield per plant. It was found by López-Sesé and Staub (2002) that the effect of combining ability on yield and its components continued for many years. According to Feyzian et al. (2009) while dominant genes are effective on high yield, the effect of new genes obtained by hybridization on the average weight of fruit was quite high too. The transfer of superior genes by hybridization (addition) increases starch content and especially seed production (in pumpkin) by repeated hybridization reported by Sanin et al. (2014) recently. The effect of agglutinative and non-agglutinative genes against the resistance to parthenocarpy and water melon mosaic virus was significant in summer squash (Douglas et al., 2011). In a study conducted with 28 *Luffa acutangula* (Roxb.) L. genotypes, very high level of genetic variation was found; the general and specific combining ability values were quite important for yield and antioxidants (ascorbic acid, total carotenoid and total phenolics), and a high mean square value for the parents and hybrids were reported (Pradip et al., 2013). Pradip et al.'s study (2013) shows similarities to our study regarding square means. In the melon study by Iathet and Piluek (2006), it was determined that number of fruit per plant and plant productivity/yield was highly genetic, and there was a high and positive correlation between the number of fruit per plant and plant yield. In a study with cucumber in the gourd family, it was recorded that the number of fruit per plant and plant yield was mostly under genetic control, while with the maximum genetic difference, the plant yield increases when number of fruit per plant increases (Mishra et al., 2007). Gabr (2003) and Abd El-Hadi et al. (2004) who worked on heterosis and economic implementations in squash, and Abdein (2005), Al-Ballat (2008) and Al-Araby (2010), found significant effects of heterosis on squash, as in our study. Thangamani et al. (2011), who conducted heterosis studies on 10 parents and diallel hybrids, reported that heterosis in squash had a positive effect on yield and quality. The study conducted by Jahan et al. (2012) determined a desired positive effect of heterosis on the number of fruit per plant and yield of pumpkins (*Cucurbita moschata* Duch. ex Poir). Marie et al. (2012), who reached results parallel to our study, found that heterosis had a high effect on yield, yield components and female flowering time.

In order to find the genetic similarities of eight domestic summer squash genotypes used in the study (Tutluca 1, Tutluca 2, Halfeti 1, Halfeti 2, Bozova Mrkz., Yaslıca, Konak and Şanlıurfa Mrkz.), a nucleotid series of the *trn*L-F region of chloroplast genome, which is widely used in genetic relationship studies in plants, is used. The alignment process, which is the most important of the processes carried out to determine

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the relationships between genotypes, was run through ClustalX 2.0, and results given in *Fig. 1*. When we look at the result of alignment, a sufficient polymorphism was seen between genotypes.

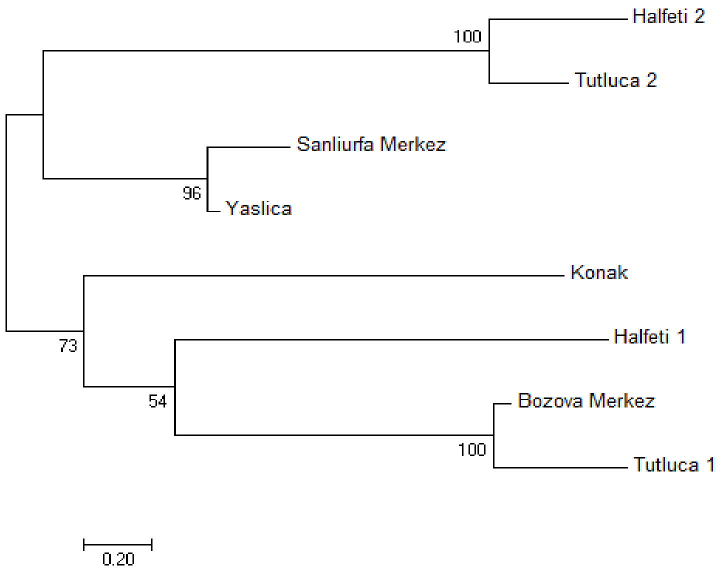

Figure 1*. The determination of the genetic relationships of the parent genotypes with the Neighbor-Joining (NJ) method (numbers on branch demonstrate the bootstrap values)*

The phylogenetic tree which was obtained with MEGA 7 (Yadav et al., 2015) software to find genetic relationships between parents was shown in *Fig. 2*. By using 500 bootstrap recurrences of the series of chloroplast *trn*L-F region, the parents were observed to be divided into two main groups (*Fig. 2*). Halfeti2, Tutluca2, Şanlıurfa Merkez and Yaslica genotypes took part in the first group. In this group, while Halfeti2 showed a close relationship with Tutluca2 with a high bootstrap value, Şanlıurfa Mrkz was also observed with almost the same level of bootstrap value and showed close relationship with the Yaslıca genotype. On the other hand, Konak, Halfeti1, Bozova Merkez, Tutluca1 genotypes took place in the second group. In this group, while Konak and Halfeti1 genotypes presented a polyphyletic situation, with a quite high bootstrap value, the Bozova Merkez and Tutluca1 genotypes were in close genetic relation and showed a monophyletic situation (*Fig. 2*).

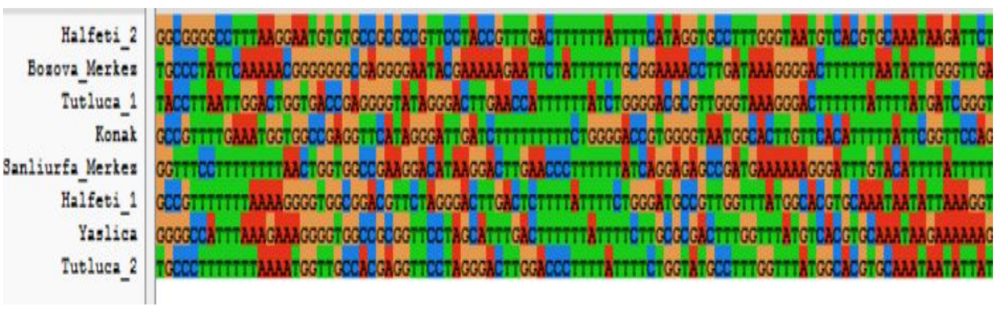

Figure 2*. A section of the series aligned with Clustal X 2.0 of parent genotypes*

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To estimate the evolutionary distance between the *trn*L-F series to determine the genetic relation between parents, the Maximum Composite Likelihood model combined with MEGA 7.0 software was used (Tamura et al.**,** 2007) (*Fig. 3*). Composite likelihood algorithm provides accurate substitution parameters and can use the pairwise distances of sequences in a distance matrix that have connection with phylogenetic relationships among the sequences (*Fig. 1*). So, our result, in which base change models between series was considered, supports the topology of the phylogenetic tree in *Fig. 1*.

	1	2	3	4	5	6	7	8
1. Halfeti 2		0.51	0.58	0.65	0.42	0.62	0.46	0.06
2. Bozova Merkez	2.74		0.04	0.49	0.37	0.52	0.46	0.62
3. Tutluca 1	3.33	0.44		0.48	0.57	0.43	0.61	0.61
4. Konak	3.73	2.75	2.65		0.46	0.57	0.35	0.55
5. Sanliurfa Merkez	2.40	2.16	2.89	2.56		0.45	0.03	0.32
6. Halfeti 1	3.59	2.49	2.33	3.03	2.57		0.31	0.63
7. Yaslica	2.65	2.45	3.15	2.03	0.27	1.89		0.32
8. Tutluca 2	0.63	3.32	3.74	3.06	1.94	3.42	1.92	

Figure 3*. The estimation of evolutionary distance between parents*

In conclusion, as a result of this study, in which eight parents and 28 hybrids (obtained from these parents without reciprocals) were used as materials, the heterotic effects of summer squash for yield and some agronomic traits were evaluated. The highest heterotic effects occurred in the summer squash yield characteristic. A maximum of 32.67% heterosis and 24.36% heterobeltiosis values were observed. With these results, it was concluded that the 5 x 8 (Bozova Mrkz x Şanlıurfa Mrkz) and the 4 x 8 (Halfeti-2 x Şanlıurfa Mrkz) hybrids are likely to be the most promising hybrid combinations for the traits analyzed, when we evaluated the parents according to their contribution to heterosis and heterobeltiosis. It was seen that the highest contribution in the summer squash yield was obtained from the Şanlıurfa Mrkz parent and followed by Konak and Yaslıca parents, respectively. Chloroplast DNA based phylogenetic relationships of parents supported that obtained values of heterosis and heterobeltiosis are permanent and not coincidental.

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APPENDIX

Chart 1. *Average values of parents and F1 hybrids in terms of the analyzed characteristics*

Parents	Plant length (cm)	Stem diameter (mm)	Plant width-spread (cm)	Leaf number (number)	50% Female flowering time (day)	Stem length (cm)	Productivity (kg/da)
1. Tutluca1	25.4	19.1	123.3	43.2	34.1	32.2	850
2. Tutluca2	28.1	18.6	120.8	41.2	36.5	34.5	910
3. Halfeti1	33.3	22.2	165.2	47.2	24.2	29.3	2380
4. Halfeti2	31.1	21.5	143.6	42.3	22.4	28.9	2080
5. Bozova Mr	18.2	15.8	116.5	39.8	28.5	36.7	900
6. Yaslıca	23.5	16.2	132.6	42.4	21.9	33.3	930
7. Konak	34.2	20.1	150.4	54.3	29.1	34.6	2440
8. Şanlıurfa Mrkz.	38.5	22.8	183.2	67.3	26.2	29.9	2600

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Hybrids							
1x2	26.57	18.68	117.52	39.83	36.26	30.67	852.90
1x3	30.17	21.36	152.15	51.15	34.75	31.80	1713.03
1x4	30.06	19.74	132.96	46.97	28.16	37.66	1549.24
1x5	23.09	19.01	123.98	43.19	29.40	29.91	883.84
1x6	25.25	19.25	123.45	46.49	24.91	40.99	914.65
1x7	28.61	19.26	147.36	50.11	33.49	36.01	1995.06
1x8	31.53	21.43	171.84	62.33	31.57	37.02	2126.24
2x3	32.97	20.22	150.69	45.71	31.46	31.65	1767.88
2x4	30.12	19.33	129.08	39.21	30.80	33.84	1550.46
2x5	23.68	17.99	114.20	42.08	31.52	30.04	1067.63
2x6	27.76	16.87	133.63	45.57	33.00	35.96	1022.12
2x7	29.31	21.01	128.71	52.61	34.28	36.18	2047.35
2x8	36.16	22.09	176.59	57.53	31.03	36.77	2229.38
3x4	33.29	23.14	142.00	52.18	26.49	22.52	1945.45
3x5	24.77	19.74	142.88	49.07	27.25	31.81	1424.01
3x6	29.62	18.33	143.27	41.64	24.67	33.41	1700.68
3x7	35.94	22.78	164.19	50.26	25.63	39.24	2928.63
3x8	38.74	23.96	193.50	60.50	19.85	34.70	3108.27
4x5	25.61	20.35	134.29	41.88	26.74	33.93	1520.25
4x6	28.47	19.51	127.29	42.08	24.84	32.88	1838.81
4x7	32.37	20.60	138.08	52.08	26.46	34.62	2652.34
4x8	37.86	23.90	185.67	59.37	24.07	37.27	2977.65
5x6	20.29	16.77	120.70	38.59	26.04	32.57	939.80
5x7	26.92	18.64	141.48	51.20	30.20	38.46	1972.77
5x8	31.07	20.07	166.30	55.20	26.30	39.87	2321.73
6x7	29.39	18.33	133.00	46.96	26.53	36.61	2008.69
6x8	21.02	11.95	180.10	64.70	23.17	36.10	2236.43
7x8	37.38	20.66	181.75	72.43	25.36	40.33	3233.41

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CV (%)	17.5	13.5	6.3	15.3	15.6	15.1	12.3
F	3.09**	2.46^{NS}	18.32**	11.18**	2.90**	59.07**	30.20**
Parents Average	29.04	19.54	141.95	47.21	27.86	32.43	1636.25
F1 Average	29.57	19.82	146.31	50.03	28.37	34.74	1876.02

**; P<0,01

Chart 2. *Heterocyst values of hybrids.*

	Plant length (cm)	Stem diameter (mm)	Plant width-spread (cm)	Leaf number (number)	50% Female flowering time (day)	Stem length (cm)	Productivity (kg/da)
1x2	-0.67	-0.88	-3.71	-5.61	2.71	-8.03	-3.08
1x3	2.79	3.43	5.48	13.17	19.21	3.41	6.07
1x4	6.40	-2.74	-0.37	9.86	-0.31	23.28	5.75
1x5	5.90	8.96	3.40	4.08	-6.07	-13.17	1.01
1x6	3.29	9.07	-3.52	8.63	-11.04	25.17	2.77
1x7	-3.98	-1.75	7.68	2.78	5.98	7.80	21.28
1x8	-1.30	2.30	12.13	12.82	4.71	19.24	23.26
2x3	7.40	-0.89	5.38	3.41	3.66	-0.78	7.47
2x4	1.76	-3.59	-2.36	-6.09	4.58	6.75	3.71
2x5	2.30	4.58	-3.75	3.91	-3.01	-15.63	17.97
2x6	7.60	-3.03	5.47	9.03	13.03	6.07	11.10
2x7	-5.90	8.56	-5.08	10.17	4.50	4.73	22.23
2x8	8.60	6.73	16.18	6.05	-1.02	14.19	27.03
3x4	3.40	5.91	-8.03	16.60	13.67	-22.62	-12.76
3x5	-3.80	3.87	1.44	12.81	3.40	-3.60	-13.17
3x6	4.30	-4.51	-3.78	-7.05	7.04	6.73	2.76
3x7	6.50	7.69	4.05	-0.96	-3.84	22.83	21.52
3x8	7.90	6.48	11.08	5.68	-21.25	17.23	24.83
4x5	3.90	9.13	3.26	2.02	5.08	3.43	2.03

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4x6	4.30	3.48	-7.83	-0.63	12.13	5.73	22.18
4x7	-0.87	-0.97	-6.07	7.83	2.76	9.03	17.36
4x8	8.80	7.91	13.63	8.34	-0.93	26.78	27.25
5x6	-2.70	4.83	-3.09	-6.10	3.33	-6.93	2.71
5x7	2.74	3.86	6.02	8.83	4.87	7.87	18.13
5x8	9.60	4.01	10.98	3.09	-3.83	19.72	32.67
6x7	1.87	1.01	-6.01	-2.87	4.04	7.83	19.21
6x8	9.20	4.83	14.06	17.96	-3.67	14.25	26.71
7x8	2.83	-3.69	8.96	19.13	-8.28	26.06	28.31
Average	3.29	3.02	2.70	5.60	1.84	7.41	13.08

Chart 3. *Heterobeltiosis values of hybrids*

	Plant length (cm)	**Stem diameter (mm)**	**Plant width-spread (cm)**	**Leaf number (number)**	**50% Female flowering time (day)**	**Stem length (cm)**	**Productivity (kg/da)**
1x2	-30.99	-18.05	-35.85	-40.81	-0.67	-16.43	-67.20
1x3	-21.64	-6.32	-16.95	-23.99	-4.80	-13.36	-34.11
1x4	-21.93	-13.40	-27.43	-30.22	-22.84	2.62	-40.41
1x5	-40.04	-16.61	-32.33	-35.82	-19.45	-18.49	-66.01
1x6	-34.40	-15.57	-32.62	-30.92	-31.76	11.70	-64.82
1x7	-25.68	-15.54	-19.56	-25.55	-8.25	-1.89	-23.27
1x8	-18.09	-6.00	-6.20	-7.38	-13.51	0.88	-18.22
2x3	-14.36	-11.32	-17.74	-32.08	-13.81	-13.76	-32.00
2x4	-21.76	-15.22	-29.54	-41.74	-15.62	-7.79	-40.37
2x5	-38.49	-21.11	-37.66	-37.47	-13.64	-18.16	-58.94
2x6	-27.89	-26.00	-27.06	-32.28	-9.58	-2.02	-60.69
2x7	-23.86	-7.87	-29.74	-21.83	-6.09	-1.41	-21.26
2x8	-6.07	-3.10	-3.61	-14.51	-14.99	0.19	-14.25
3x4	-13.52	1.50	-22.49	-22.47	-27.44	-38.64	-25.17

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3x5	-35.66	-13.44	-22.01	-27.08	-25.35	-13.32	-45.23
3x6	-23.06	-19.59	-21.79	-38.13	-32.40	-8.97	-34.59
3x7	-6.64	-0.10	-10.38	-25.32	-29.79	6.93	12.64
3x8	0.61	5.08	5.62	-10.10	-45.63	-5.45	19.55
4x5	-33.48	-10.73	-26.70	-37.77	-26.73	-7.56	-41.53
4x6	-26.04	-14.45	-30.52	-37.47	-31.95	-10.40	-29.28
4x7	-15.93	-9.66	-24.63	-22.61	-27.50	-5.68	2.01
4x8	-1.66	4.83	1.35	-11.78	-34.04	1.56	14.53
5x6	-47.31	-26.44	-34.11	-42.66	-28.66	-11.24	-63.85
5x7	-30.08	-18.23	-22.77	-23.92	-17.25	4.78	-24.12
5x8	-19.29	-11.96	-9.22	-17.97	-27.94	8.63	-10.70
6x7	-23.66	-19.59	-27.40	-30.22	-27.31	-0.25	-22.74
6x8	-45.40	-47.59	-1.69	-3.86	-36.53	-1.63	-13.98
7x8	-2.91	-9.39	-0.79	7.62	-30.52	9.90	24.36
Average	-23.19	-13.07	-20.14	-25.66	-22.29	-5.33	-27.85

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DYNAMICS OF SEXUAL REGENERATION IN THREE NATIVE OAK SPECIES (*QUERCUS BRANTII* LINDL., *Q. INFECTORIA* OLIV., AND *Q. LIBANI* OLIV.) OF ZAGROS FORESTS, IRAN

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Abstract. Zagros forests of Iran have been historically degraded by anthropogenic and other biotic and abiotic factors. As a result of multiple factors, seedlings and saplings establishment of Zagros oaks rarely happens and their dynamic is still unknown. Because of many difficulties to study the dynamics of oak sexual regeneration in Zagros forests, we focused on all of three Zagros oaks (*Quercus brantii* Lindl., *Q. infectoria* Oliv., and *Q. libani* Oliv.) planted in National Botanical Garden of Iran. We conducted 8 full-census inventories from the early September 2013 to the late September 2016 to monitor the trend of regeneration loss. The results showed that the 77.7%, 87%, and 89.8% of the total produced acorns in *Q. brantii*, *Q. infectoria* and *Q. libani* are demolished prior to germination and establishment in forest floor, respectively. Besides, only 4.4%, 3.2%, and 2.1% of produced acorns have the chance to become three-year saplings in the studied species. The results confirm the important role of wildlife in consumption of acorns and the variety in survival trends of saplings among the studied oak species. Study of sapling establishment in longer period and to conduct similar researches on other native oak species of Iran could be suggested for future studies.
Keywords: *acorn, monitoring, sapling, seedling establishment, survival*

Introduction

Historically, one of the most important challenges and responsibilities of the forest managers has been to promote regeneration in forests (Nyland et al., 2016). Acorn production plays a fundamental role in the organization, dynamics and sustainability of oak forest ecosystems (Rose et al., 2012). The abundance of acorns directly affects the regeneration of oak and the abundance of acorn-consuming animal species, and indirectly affects the predators and parasites of acorn consumers (Dey, 1995; McShea and Healy, 2002; Greenberg and Warburton, 2007).

Acorn production is characterized by extreme variation among years (Sork et al., 1993; Koenig et al., 1994a; Greenberg, 2000; Steen et al., 2009; Koenig and Knops, 2014) and individual trees (Auchmoody et al., 1993; Koenig and Knops, 1998; Gea-Izquierdo et al., 2006). The size of acorn crops affects many components of the ecosystem, and both annual and individual variation in acorn production influence the regeneration and management of oak forests. Acorn production is influenced by

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weather, insects, wildlife, tree age and size, tree crown position, and the tree`s inherent capacity to produce acorns (Dey, 1995).

Many organisms consume acorns, including insects, millipedes, fungi, birds and mammals. Consumption by one or more biotic agents is often so complete that in any given year few acorns remain to germinate and develop to seedlings. Although this consumption contributes to oak regeneration failures, acorns are valuable sources of food for many birds and mammals because of their high caloric content, nourishment and availability during seasons when other food is often scarce (Johnson et al., 2009). The term dispersal is used to refer to the transport of acorns from their place of origin (i.e. tree crowns or the forest floor) to other locations. Birds and mammals are the most important dispersers of acorns, which that nearly consume all the acorns they disperse, thus clearly causing some degree of loss to potential seedling establishment. Dispersers may also consume significant quantities of acorns at their place of origin. If proportionately few acorns are consumed by a disperser, dispersal may be advantageous to the oak if acorns are dispersed in significant number to habitats that are favorable for seedling establishment (Johnson et al., 2009).

Rodents, together with other animals, often consume most of the acorn crop following their fall. Thus, establishment of oak seedlings occur primarily in years when the amount of sound acorns exceeds the demand for acorns by insects and wildlife (Christisen and Kearby, 1984). Production of sound acorns exceeds this level only during years with good to bumper acorn crops (Beck, 1977).

Factors influencing early survival and growth of seedling beneath a stand may be very different from those determining growth of saplings. Initial survival and early growth of oak seedlings are primarily dependent on stored food reserves in the acorns. Light is a dominant environmental factor limiting seedling establishment and soil moisture may also limit establishment. Oak seedlings begin spring growth, when both light and soil moisture conditions are favorable (Johnson et al., 2009). Oaks are generally regarded as drought tolerant, whereas oak seedlings are relatively shade-intolerant. Under closed and dense canopies, oak seedlings cannot survive long (Crunkilton et al., 1992).

Quercus sp. is the most frequent genus of Fagaceae in forests of Iran (Sabeti, 2016). Oaks are the most prevalent forest types in Iran and dominate most of the Zagros landscape. Zagros forests are the most important and sensitive forest ecosystems of Iran with an area of about 6 million ha (Pourhashemi et al., 2004; Sagheb Talebi et al., 2014). Various factors such as traditional overutilization from timber and non-timber products, forest land conversion to agricultural lands, overgrazing, and road and dam constructions, have contributed to the degradation of these forests. Besides, many individual oaks are affected by decline phenomenon during the last decade. In this situation, oaks produce acorns, but seedlings and saplings are rarely established on the forest floor (Sagheb Talebi et al., 2014). All in all, the study of sexual regeneration dynamic of Zagros oaks is impossible because of degradation factors.

Although dynamics of acorn production have been studied in various researches worldwide (e.g. Koenig et al., 1994b; Greenberg, 2000; Abrahamson and Layne, 2003; Perez-Izquierdo and Pulido, 2013), a few researchers (e.g. Panahi et al., 2009; Pourhashemi et al., 2013, 2015) published studies on native oaks of Zagros region in Iran. Furthermore, there is no research on sexual regeneration dynamics of Zagros native oaks.

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During the past three decades, native oak species of Zagros forests are planted in National Botanical Garden of Iran (NBGI), for ex situ conservation. With an area of about 150 ha, the NBGI is the most important and the largest botanical garden in Iran. Today, oak species are one of the most important elements of the NBGI which are distributed throughout the garden, mainly within the Zagros collection. The mature oak individuals normally produce acorns and regenerate sexually. This garden can be considered as the unique place of Iran in which there is potential form of oak sexual regeneration. Our study of sexual regeneration dynamics has been partly enabled due to the fact that the oak collection of NBGI is protected from destruction of acorns by livestock or harvesting by humans.

Material and methods

Study area

The study was conducted at NBGI (35˚41´ N, 51˚19´ E) from 2013 to 2016. The garden was founded in 1968 in the vicinity of Tehran at an altitude of about 1320 m. The terrain is flat and slopes gently to the south. The climate is dry with an average annual precipitation of about 257 mm falling between November and May. Temperature reaches as much as 42-43ºC during July and August. During winter the temperature may fall to -10ºC or lower. The garden consists of different native and exotic collections such as Alborz, Himalayan and Rock garden (*Fig. 1a*).The Zagros collection is one of the most important forest collections of the garden that covers an area of ca. 3 ha. Most of the oak trees are found in these collections (*Fig. 1b*).

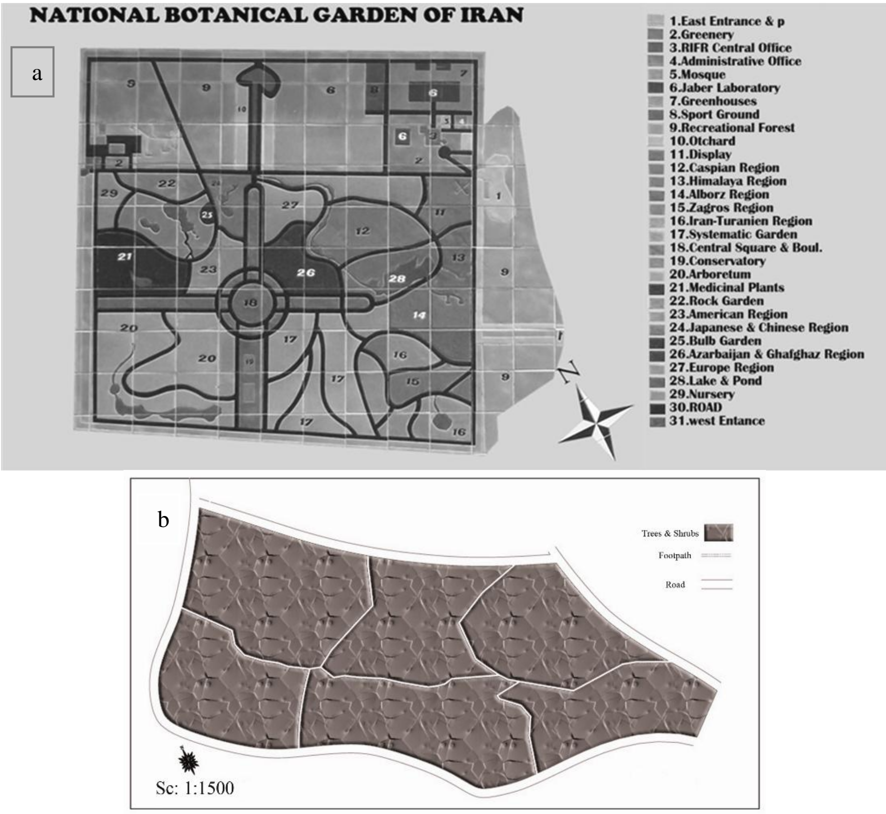

Figure 1. *Map of the NBGI (a) and Zagros collection (b)*

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The studied species

All of three main oak species of Zagros forests planted in NBGI were considered for this study, including Lebanon oak (*Q. libani* Oliv.), Aleppo oak (*Q. infectoria* Oliv.) and Brant`s oak (*Q. brantii* Lindl.). The studied oaks naturally grow in Zagros forests. *Quercus libani* is only widespread in northern Zagros (West Azerbaijan and Kurdistan provinces), while *Q. infectoria* is widely distributed in West Azerbaijan, Kurdistan, Kermanshah and Lorestan provinces. Compared to Lebanon oak, Brant`s oak is usually found on northern slopes as well but at lower elevations. *Quercus brantii* is more resilient and tolerant than the other two oak species and its geographic distribution is not limited by elevation or aspect, thus it is found at various aspects and elevations. It occurs throughout the Zagros forests, mainly in the southern and central areas (Sagheb Talebi et al., 2014).

Sampling procedure

After initial garden survey, 5 sample trees were selected for each oak species in flat areas. The selected trees were larger than 15 cm in diameter at breast height (DBH), and dominant in crown position. Furthermore, the crown of adjacent trees had no contact. Two full callipering were done in the first year of the research. In the first callipering, the number of acorns per tree was determined by crown count method (Gysel, 1956) in early September, just prior to acorn fall. The counted acorns considered as potential of the sample oak trees. To standardize comparisons among different sized trees and simplify the usage by forest management, the numbers of acorns per tree were converted to the number per m^2 crown area per tree (acorn density) by dividing the total acorn production of each tree by its crown area (Christisen and Kearby, 1984; Rose et al., 2012). The second full callipering was conducted in late December after full acorn fall and included counting the numbers of acorns beneath the sample tree crowns. The difference of acorn number between the two inventories indicates the portion of acorns which have been consumed by animals directly from the trees.

In the second year of the research, two other full callipering (termed as third and fourth inventories) were carried out in the first (early May) and the end (late September) of the growing season, in which all of the established seedlings under the sample tree crowns were counted. The differences of acorns and seedlings number between second and third inventories indicate portion of the acorns that could pass the winter and transform to the seedlings in the next spring. The differences of seedlings between the beginning and the end of growing season show the portion of seedlings that could pass the summer. The same inventory procedure was done in the third and fourth years of the study to determine the losses of 2-year and three-year saplings.

Statistical analysis

The rate of survival was initially calculated for each species. For comparing the losses of different inventory stages, the numbers of acorns, seedlings and saplings were converted to percent. Normal distribution of variables was assessed by test of Shapiro-Wilk test ($p < 0.01$) and the homogeneity of variances was assessed by Levene test. The acorn density was natural-log transformed for ANOVA to reduce the correlation between the mean and variance (Sakal and Rohlf, 2012). The differences of acorn, seedling and sapling abundances in each species were tested by non-parametric

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Kruskal- Wallis test. In addition, pairwise comparison was done by Mann- Whitney U test. Statistical significance was reported at the p < 0.05 level unless otherwise stated.

Results

Detailed quantitative characteristics of sample trees are summarized in *Table 1*. Based on one-way ANOVA analysis, there was no significance difference among the studied species regarding to acorn density (df = 2, p < 0.061, F = 3.568). The numbers of acorns produced by sample trees are shown in *Fig. 2*.

Table 1. *Quantitative characteristics of sample trees*

Variable	*Q. brantii*		*Q. infectoria*		*Q. libani*	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
Diameter at breast height (cm)	27.6	21-34	27	22-34	23.6	19-27
Crown area (m^2)	33.9	26.9-41.3	27.5	20.4-36.3	23.3	15.5-30.7
Number of acorns per tree	758.4	228-2020	1597.4	700-3032	801.8	204-1453
Number of acorns per m^2 crown	21	7.6-51	63.4	30.6-148.5	34	9.1-65.9

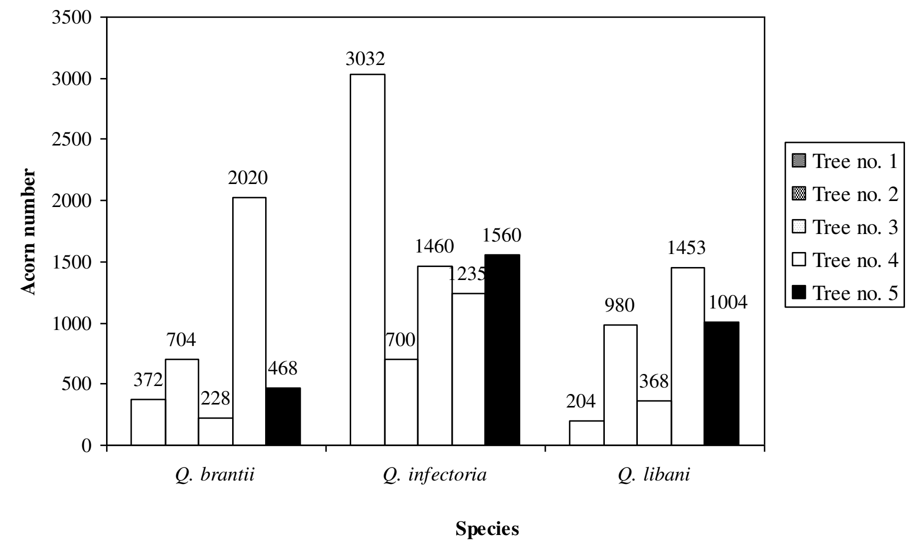

Figure 2. *Number of acorns of the studied oak species*

Losses of sexual regeneration from acorn to three-year saplings for each species are shown in *Fig. 3*. Totally, three main trends were observed as follows:

- Some individual trees produced large number of acorns (more than 1500) and the losses were moderately sever or sever. The losses of these individual trees were more than 90 percent (e.g. tree no. 4 in all three studied oak species and tree no. 1 in *Q. infectoria*).
- Some individuals produced moderate number of acorns (between 500 to 1500) and the losses were moderate, too (e.g. trees no. 2 in both of *Q. brantii* and *Q. infectoria* and trees no. 2 and 5 in *Q. libani*).
- Some individuals produced low number of acorns (less than 500) and the losses were moderate (e.g. trees no. 1 and 3 in both of *Q. brantii* and *Q. libani*).

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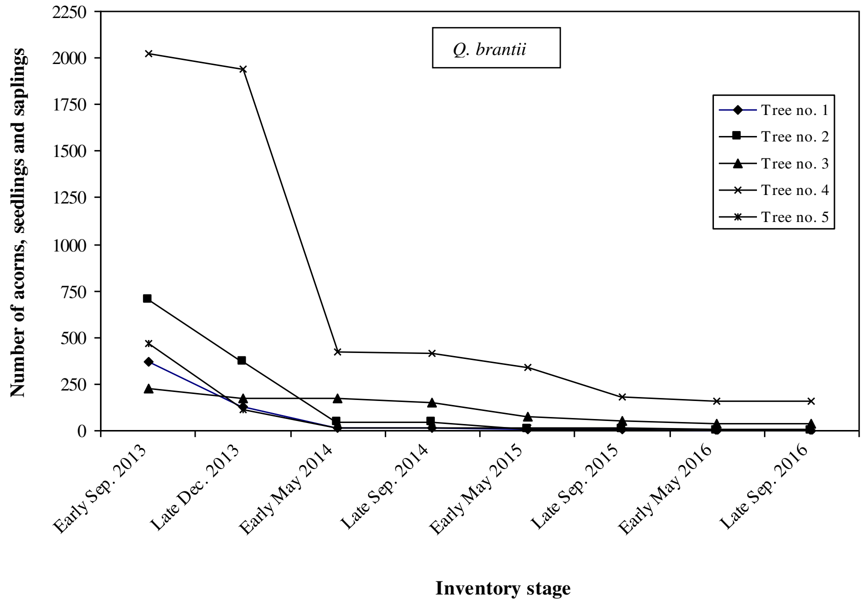
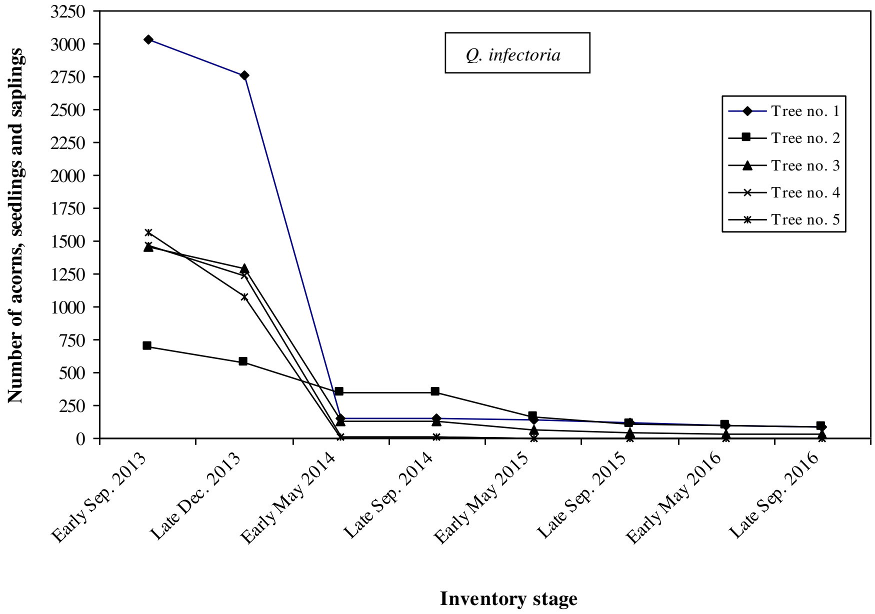
There were some considerable cases among the sample trees. For instance, the acorn production potential of tree no. 1 in *Q. infectoria* was remarkably more than the others due to its 3032 produced acorns. Another exceptional case was observed in tree no. 5 in *Q. libani*. While it produced 1004 acorns, no established seedlings were observed beneath its crown.

Survival percentage showed no significance difference among studied species (χ^2 = 0.384; p = 0.825) and among same inventory stages (*Fig. 4*). In other words, the three studied oaks had similar rates of loss in same inventory stages, but the differences among inventory stages in each species was significant (*Q. brantii*: df = 6, χ^2 = 19.49, p = 0.002; *Q. infectoria*: df = 6, χ^2 = 21.216, p = 0.001; *Q. libani*: df = 6, χ^2 = 21.997, p = 0.001).





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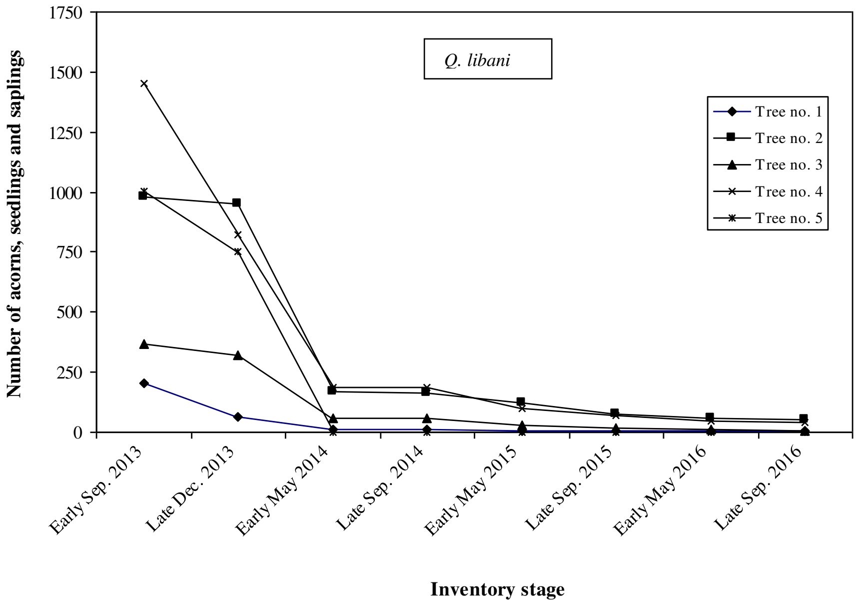

Figure 3. *The proportion of the studied oak acorns that produce seedlings and saplings*

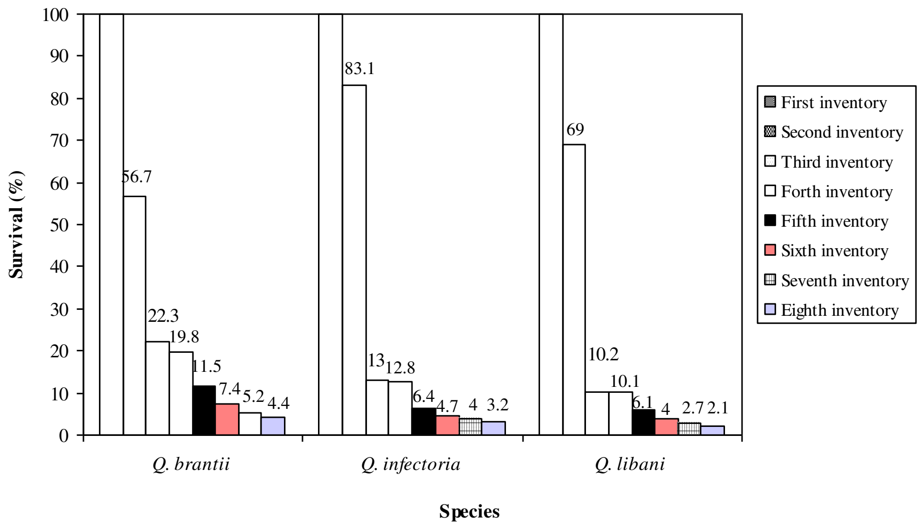

Figure 4. *The survival (percent) from acorn to three-year sapling in the studied species (results of the inventory stages comparison: First inventory: df = 2, $\chi^2 = 0$, p = 1; second inventory: df = 2, $\chi^2 = 2$, p = 0.368; third inventory: df = 2, $\chi^2 = 0.62$, p = 0.733; forth inventory: df = 2, $\chi^2 = 0.62$, p = 0.733; fifth inventory: df = 2, $\chi^2 = 0.26$, p = 0.787; sixth inventory: df = 2, $\chi^2 = 0.32$, p = 0.852; seventh inventory: df = 2, $\chi^2 = 0.28$, p = 0.82; eighth inventory: df = 2, $\chi^2 = 0.22$, p = 0.832)*

Based on the pairwise comparison of the losses in inventory stages, significant difference was observed after the second inventory stage in *Q. brantii*. However, the same condition did not hold true after the third inventory stage in *Q. infectoria* and *Q. libani* (*Figs. 5, 6*).

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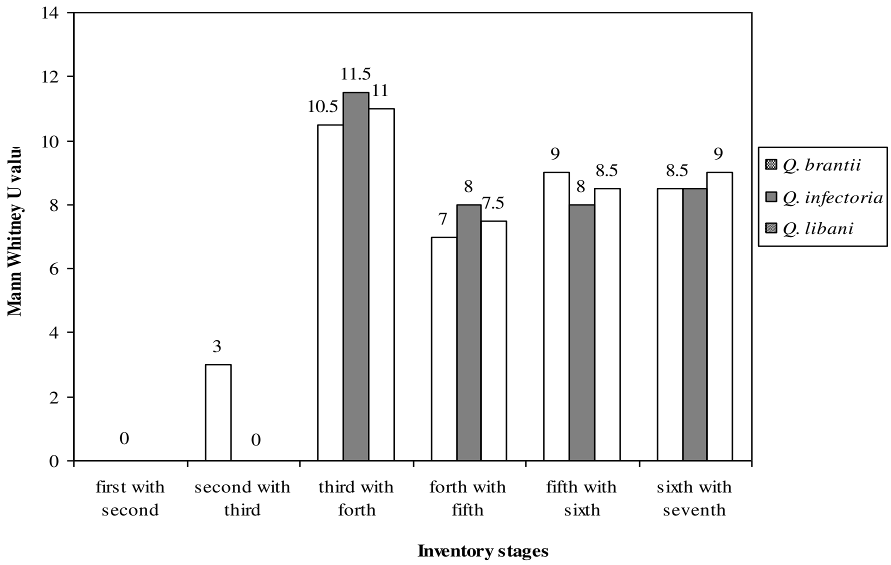

Figure 5. *Mann-Whitney U values of pairwise comparison of the inventory stages in the studied species (The losses percent between the first and second stages of inventory was considered as the first inventory stage)*

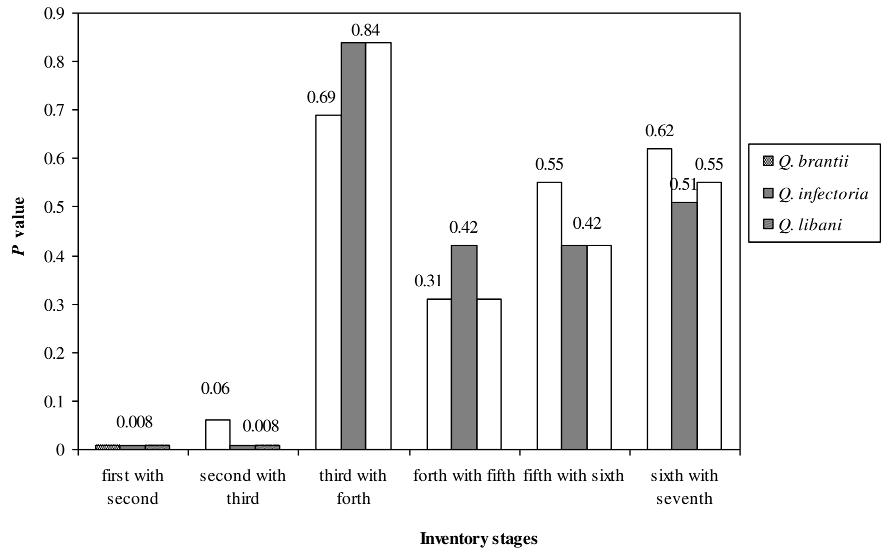

Figure 6*. P values of pairwise comparison of the inventory stages in the studied species (The losses percent between the first and second stages of inventory was considered as the first inventory stage)*

Discussion

Our results confirm that acorn production significantly vary from tree-to-tree for each oak species. Some researchers have emphasized this variation in oaks (e.g. Greenberg, 2000; Rose et al., 2012; Koenig and Knops, 2014). All our sample trees were similar regarding to the DBH, crown area, crown position, and environmental factors. Therefore, the differences of acorn production among studied species can be attributed to inherent characteristics of the trees. Although the tree no. 4 in *Q. infectoria* showed a high rate of acorn production, it was difficult to consider it as an inherently

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good producer without long-term monitoring of acorn production. On the other hand, the two-year maturation of acorns in *Q. libani* and *Q. brantii* (Djavanchir Khoie, 1967) might have been affected by the climatic factors during the year before the first year of the study.

The average number of acorns in *Q. brantii*, *Q. infectoria* and *Q. libani* were 21, 63.4 and 34, respectively. In a same research in the NBGI, mean values of acorn production were 46.5, 52.4, and 31 in 2006 (Panahi et al., 2009). The differences of acorn production between 2006 and 2013 confirm the year-to-year variability of acorn production in the same species, which had been the subject of a number of studies (e.g. Steen et al., 2009; Koenig and Knops, 2014). One may also note that the number of sample trees and their quantitative variables could affect the average number of produced acorn.

Losses of acorn production due to insects, birds and mammals can be very high. Although the role of insects is very important in destroying the acorns, no damages were observed in the studied oak species in the NBGI, which is obviously due to the conservation status of the garden. About 10-25% of acorns are taken directly from the trees by animals such as birds and squirrels (Beck, 1977; Myers, 1978). Our results showed the remarkable rate of consumption of acorns by rodents especially squirrels, and birds especially crows, woodpeckers, and rabbits. The maximum consumption of acorns directly from the trees occurred in *Q. brantii* (43.3% of the total produced acorns), while the values for *Q. infectoria* and *Q. libani* were 17.1% and 31%, respectively. Totally, the portion of acorns taken directly from the trees was more than the average global range of 10-25%. Of course the bigger size of acorns in *Q. brantii* and *Q. libani* in comparison with *Q. infectoria* (Djavanchir Khoie, 1967) can be further reason for higher consumption rate. It is not clear whether seedling establishment is positively related to acorn size (Sork, 1993; Díaz et al., 2003). The role of acorn size is controversial: bigger acorns are preferably consumed by predators (Gómez, 2004), which might hamper regeneration. On the other hand, seedlings from bigger acorns are associated with an average higher number of leaves and leaf area (Díaz et al., 2003), which might be an advantage for young seedlings.

As a general rule of overwintering, acorns require protection from desiccation, low temperatures and predation by insects, rodents and other animals (Johnson et al., 2009). The same applied for the losses of acorns from early September 2013 to late December 2013, as the proportion of acorns consumed by wildlife from ground surface during winter was high (34.4%, 70.1%, and 58.8% of the total produced acorns in *Q. brantii*, *Q. infectoria* and *Q. libani*, respectively; *Fig. 4*). Obviously a minor portion of this loss could be related to the low viability of acorns in the studied species. High rate of acorn consumption by wildlife in the garden, both directly from the crown and from forest floor, indicate their important role in sexual regeneration chain of oaks. Totally, 77.7%, 87%, and 89.8% of total produced acorns were destroyed prior to the spring of 2014 in *Q. brantii*, *Q. infectoria* and *Q. libani*, respectively (*Fig. 4*). In *Q. brantii*, significant difference amongst losses was observed in the interval between the first and second inventory stages (*Fig. 6*), but the situation was a little different in *Q. infectoria* and *Q. libani*. Furthermore, in these species the losses were significantly different during winter after acorn fall. The loss trends for all of the studied species decreased after the third inventory stage (*Fig. 4*).

Based on our garden surveys, the canopy was closed with minor gaps, though the crowns of the studied oak trees had no overlap or contact. On the other hand, soil

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moisture in the garden cannot be considered as a limiting factor because of the irrigation of the garden during dry seasons. It seems that the role of light is very important in germination and early growth of acorns in the garden.

Our results showed that the mortality rate of saplings in *Q. libani* was higher (81.7%) than the others (74.5% in *Q. brantii* and 74.9% in *Q. infectoria*). Excessive shade and competition are the most important reasons for the slow growth and mortality of saplings. The ability to persist under shade appears to vary among oak species (Lormier, 1992), which was also confirmed by our results.

Conclusion

This study mainly confirms the role of botanical gardens as appropriate sites for studying subjects that are impossible to be studied elsewhere. Besides, absence of degradation factors parallel with conservation of oak species provides an ideal condition to study the potential of trees. As previously mentioned, due to multiple reasons it is not possible to study the sexual regeneration dynamics of oak species in natural Zagros forests of Iran, including traditional utilization of acorns by forest residents, livestock grazing, and rain-fed agriculture underneath the trees. Finally, study on annual variation of acorn production, the effect of different factors on acorn production, and advantages and disadvantages of rodents and birds (as the most important acorn consumers) are the main motivations for further research.

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SOCIOECONOMIC DRIVERS OF SPATIO-TEMPORAL LAND USE/LAND COVER CHANGES IN A RAPIDLY URBANIZING AREA OF CHINA, THE SU-XI-CHANG REGION

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Abstract. The rapid economic development resulting from Chinese economic reform has greatly accelerated urbanization and industrialization, thus leading to alterations in the natural landscape. Understanding the extent of these changes is important for regional sustainable land management. An integrated approach with a geographic information system and remote sensing was used to extract land use/land cover (LULC) change information for the Su-Xi-Chang region over the period of 1980 to 2010. To calculate the dimensions of fragmentation and to observe changes in spatial patterns, FRAGSTATS was used. Major drivers were determined through bivariate statistical analysis of socioeconomic data sources. Three change matrices were constructed for detecting LULC changes from 1980 to 1990, 1990 to 2000, and 2000 to 2010. In the study period, farmland, water bodies and wetlands significantly decreased. However, construction land, grassland and woodlands increased considerably. The pattern and composition of the landscape exhibited significant fragmentation for the whole area. Socioeconomic analysis showed that population growth and economic development, urbanization and subsequent construction land expansion, and industrialization were the major socioeconomic drivers of LULC changes. Changes in farmland resulted in decreases in area and production that might impair ecological functions and decrease food production. Therefore, better land use policy would address the consequences of the loss of the natural landscape due to drastic socioeconomic drivers in the region. Further research along these lines should be encouraged, because additional studies will be helpful for the decision makers engaged in planning activities at various levels.
Keywords: *GIS, temporal changes, landscape ecology, urbanization, landscape pattern*

Introduction

A wide range of drivers associated with social, biophysical, environmental, economic and technological factors lead to land use/land cover (LULC) changes in different parts of the world (Beilin et al., 2014; Tian, 2015). The drivers of these changes may be well known, such as demographic change (Salvati et al., 2017), industrial development (Li et

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al., 2010), agricultural expansion (Kibret et al., 2016), urbanization (Deng et al., 2015; Xian and Crane, 2005), global market forces (Temesgen et al., 2013), and climatological change, such as drought and rainfall variability (Amuti and Luo, 2014; Biazin and Sterk, 2013; Luo and Zhang, 2014; Román-Cuesta et al., 2014), or they may also involve interactions of institutional or cultural impacts (Kindu et al., 2015; Sakayarote and Shrestha, 2017). These drivers have triggered drastic LULC conversions by substituting one type of LULC with another. Through these processes, the pattern, structure and function of the natural landscape are inevitably altered (Lambin et al., 2001), thus consequently influencing the global environment (Amuti and Luo, 2014). Particularly, the socioeconomic drivers are more intense than the physical drivers of these processes (Desalegn et al., 2014). Understanding these factors is vital for forecasting future LULC dynamics by using models (Lambin et al., 2001) or for the design of management strategies and policies for the sustainable management of land resources (Mottet et al., 2006; Turner et al., 1993).

Since the launch of economic reform and the opening-up policy in the late 1970s in China, LULC changes have rapidly taken place, and land use patterns are quite spatially variable throughout the country (Hua et al., 2010; Wei and Ye, 2009). Particularly since the beginning of the 21st century, the rapid socioeconomic development has resulted in remarkable changes in the spatio-temporal distributions of LULC and has adversely affected the natural environment (Deng et al., 2015; Yirsaw et al., 2016). However, because of the diversified and complex driving forces in the country, there are substantial arguments about the extent and magnitude of changes in LULC (Xu, 2004). Previous studies have revealed different findings on LULC change drivers in different regions of the country. For instance, Wang et al. (2011) have suggested the expansion of residential areas to cause LULC changes at the expense of agricultural land in northeastern China. Long et al. (2009) have proposed that rapid economic developments are major drivers for the process. Li et al. (2010) have claimed that in the eastern fringe of the Tibetan Plateau of China, the changes have occurred because of climate change and human activities. Kuang et al. (2016) have suggested that the expansion of industrialization is an important factor in LULC change in eastern China. Other studies have indicated that land policy is a major influential driver in LULC changes (Liu et al., 2010b).

Though substantial efforts have been made to identify factors driving LULC changes in different parts of the country, those research findings have revealed that the drivers vary from place to place depending on location-specific factors. Moreover, there is significant disagreement regarding the extent of influence by these drivers of changes, thus making generalizations nearly impossible (Li and Yeh, 2004; Wu et al., 2013). Therefore, any intervention to address drivers of change for sustainable land management must begin with locally specific understanding of the different drivers affecting the LULC. This requirement is especially true for the Su-Xi-Chang region of eastern coastal China, which has a fragile environment where substantial LULC changes have occurred. In the Su-Xi-Chang region, as stated by Zhou et al. (2014), owing to economic development, different conflicts, such as the conflict between land resources and demand for development versus the vulnerability of the coastal area, have arisen. Moreover, Xie et al. (2007) have suggested that both the existence of rapid economic development and the loose and neglected control of land use have put the LULC management of the region at risk. Thus, it is crucial to determine the dynamics of LULC and the main socioeconomic drivers of the change in this fragile environment. This

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information would facilitate measures to arrest and reverse the situation of LULC changes in the region and would improve understanding of the influence of socioeconomic drivers on LULC changes.

Considering these factors, this paper was designed with the following objectives: (1) to characterize land use dynamics and patterns of LULC change across the study area over the past three decades; (2) to determine the major socioeconomic drivers in periods from 1980 to 2010; and (3) to assess the relationship between LULC changes and the major socioeconomic drivers.

Materials and Methods

Study area

Encompassing three municipalities, Suzhou, Wuxi and Changzhou, the Su-Xi-Chang region is located in the Jiangsu Province ($36^0$46’ – $32^0$04’N, $119^0$08’–$121^0$15’E) in the middle of the Yangtze River Delta Economic zone of east China. It covers a total area of 1.7×10^4 km^2, with an average elevation below 50 meters (*Fig. 1*). The area has a monsoon climate with a mean annual temperature and precipitation of 15.3^0C and 1,092 mm, respectively (Long et al., 2009).

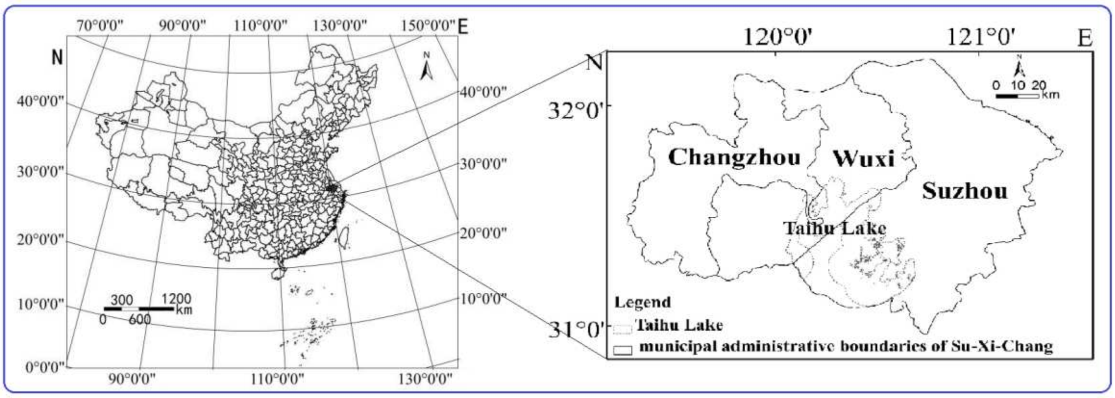

Figure 1. *Location of the study area*

In 2014, the region had a total population of 14.55 million (838/km^2), which was more than 6 times the total population per km^2 (135.5/km^2) of the country in the same year. Similarly, the GDP per capita of the Su-Xi-Chang region (123,325 CNY) was three times more than the GDP per capita of the country in the same period (NBSC, 2014). From the early 1980s, the growth of Shanghai has pushed the region toward rapid urbanization and industrialization (Long et al., 2009). Unprecedented changes in the local economy have taken place in recent decades. On the grounds of diversified cooperative enterprises in the mid-1980s, the region has served as a model for the advancement of rural industrialization (Xie et al., 2007; Tan, 1986). With these changes, the Su-Xi-Chang region has confirmed its position of importance at the national level, and today, it constitutes the Key Economic Zone in Eastern China. However, with the development of economic activities, drastic changes in LULC have been occurring in the region. These changes have prompted concerns and attempts to detect and monitor

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changes in LULC and to assess the main socioeconomic drivers of the changes, in order to attain sustainable land management.

Data source and processing

For time series LULC changes analysis (1980, 1990, 2000 and 2010), the study used vectored data sets with a 30 m spatial resolution from Landsat reflectance products and the DEM of the Computer Network Information Center, Chinese Academy of Science. Additionally, administrative boundaries at the county level were used, which were provided by the Data Sharing Infrastructure of Earth System Science, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Science. MSS and TM images were geometrically corrected using ground control points, with ETM+ images as the master and reference images. To identify the agreement in classification consistency and accuracy, effective classification was conducted by using standard procedures (Liu et al., 2005). Accordingly, as shown in *Table 1,* six land use categories were classified for the four study periods: farmland, construction land, grassland, woodland, water body, and wetland. The ESRI ArcGIS spatial analysis model was applied to measure variation within LULC types. A set of pattern metrics was identified to examine cover change spatial patterns, and FRAGSTATS was used to compute the dimension of fragmentation. In addition, a time series of socioeconomic data on population, industrial output value, agricultural production (crop yield), aquatic products, GDP, agricultural land, and construction land (settlement areas) from 1980 to 2010 was collected from local government offices and statistical year books for the region (Appendix A). These data were used to analyze the potential socioeconomic driving forces triggering LULC changes in the Su-Xi-Chang region.

Methods

Mainly on the basis of classified and gridded land use polygon themes (*Fig. 2*), internal change variations were computed between each consecutive map (from 1980 to 1990, 1990 to 2000 and 2000 to 2010). For each consecutive pair of gridded time series maps, a transition matrix was assembled (*Tables 2, 3 and 4*). Then, for each LULC category m in the transition matrix, the conversion between the two periods was computed via the GIS analysis function:

$$\Delta L_m = (r_m - r_{.m})/r_{.m} \times 100 \quad \text{(Eq.1)}$$

where ΔL_m is the change of LULC in row m, relative to the previous compared year; $r_{m.}$ is the row total of grid cells for category m; and $r_{.m}$ is the column total of grid cells for category m.

For exploring the internal conversion between different cover types, we treated the change (decrease or increase) for a cover category in a given year relative to a compared year considering various “loss or gain” conversions. Thus, for any type of “conversion loss to” or “conversion gain from”, the percentage taken by this type in the total “loss or gain” was calculated as:

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$$\begin{cases} R_{loss(m)n} = \dfrac{(r_{nm} - r_{mn})}{(r_{m.} - r_{.m})} \times 100 \quad m \neq n \\ R_{gain(m)n} = \dfrac{(r_{m,n} - r_{n,m})}{(r_{m.} - r_{.m})} \times 100 \quad m \neq n \end{cases} \tag{Eq.2}$$

where $R_{loss(m)n}$ is the percentage taken by type n in the total "conversion loss" of category row m; $R_{gain(m)n}$ is the percentage taken by n in the total "conversion gain" of category row m; and r_{nm}, and r_{mn} are the individual entries in a change matrix.

Bivariate correlation statistical analysis was performed to assess the relationships between LULC changes and socioeconomic drivers (*Table 6*). Through examining the spatial change patterns, we identified a set of pattern metrics at both the class and landscape levels that capture different dimensions of land fragmentation. Parker et al. (2001) have observed the absence of a site (region) specific typical set of metrics, as a result of variations in the significance of specific metrics significance based on research objectives and study area characteristics. As indicated by (McGarigal et al., 2002), six major landscape metrics at the class level, including patch density (PD), largest patch index (LPI), number of patches (NP), landscape shape index (LSI), area-weighted mean patch fractal dimension (AWMPFD), and interspersion and juxtaposition (IJI); and six at the landscape level, including Shannon's diversity index (SHDI), number of patches (NP), largest patch index (LPI), area-weighted mean patch fractal dimension (AWMPFD), contagion index (CONTAG) and interspersion and juxtaposition (IJI), were selected for this study on the basis of our research objectives and study area characteristics. These indices were calculated using FRAGSTATS, a spatial pattern analysis program for categorical maps (*Table 5*, and *Fig. 3*).

Result and discussion

Spatial patterns of LULC dynamics

Figure 2 shows the spatial pattern of LULC changes for the years 1980, 1990, 2000 and 2010 of the study area. In 1980, farmland and water body were the dominant cover types, and the direction of urban development (collectively termed construction land) was confined to the central part of the three municipalities – Su Zhou, Wu Xi and Chang Zhou of the Su-Xi-Chang region (*Fig. 2*).

However, in 1990, construction land started to replace some of the farmland. The construction land development tended to extend further in the north and northeast directions between 1980 and 1990, when the region embarked on economic reform by developing collective enterprises at the start of the age of rapid urban and industrial development. In the 1980s, township enterprises expanded with the formation of various small towns in the region (Long et al., 2009). After 1990, construction land became more prevalent, and the pace of urbanization and industrialization was considerably accelerated. Particularly at the end of 2010, the area of construction land overtook the area of farmland (*Fig. 2*) and covered more than 29% of the studied landscape (*Table 1*). However, farmlands started to vanish at the same time, because the demand for construction land dramatically increased with socioeconomic development.

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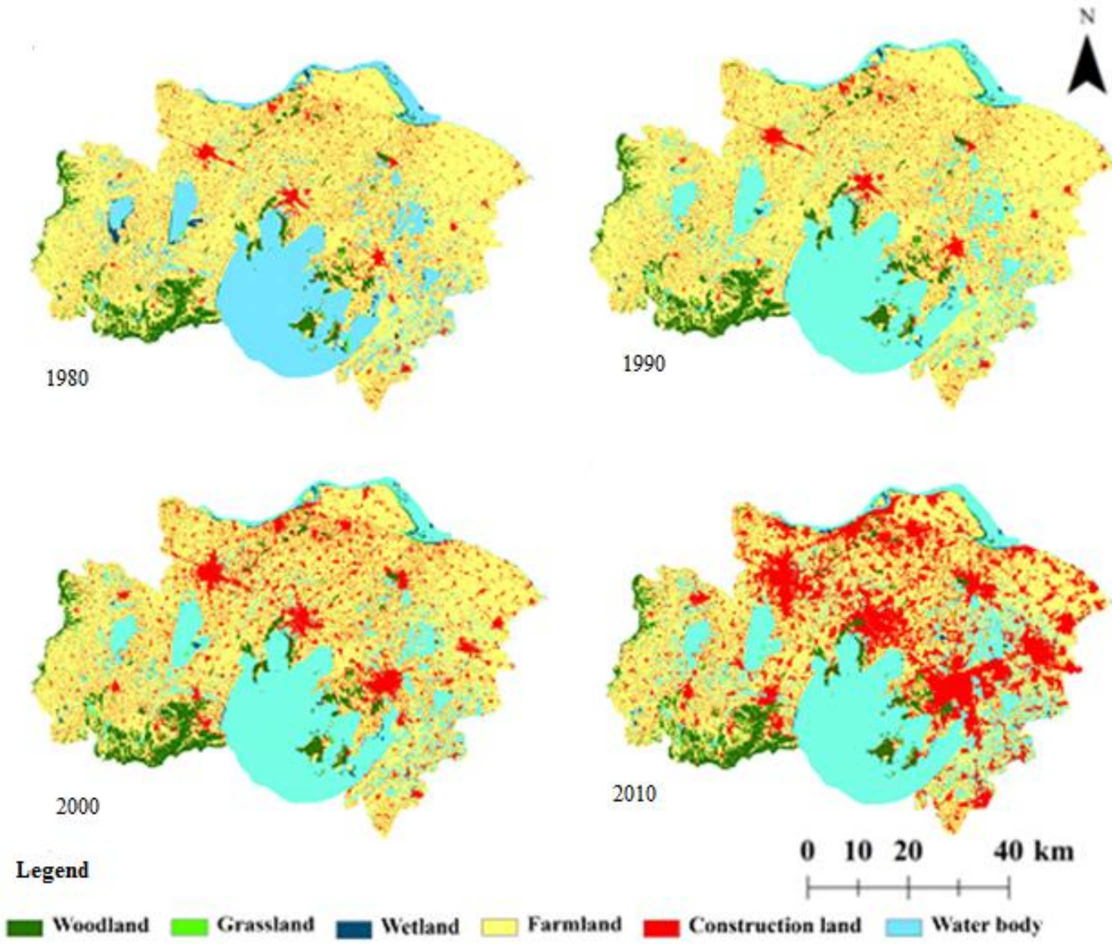

Figure 2. *LULC maps of the Su-Xi-Chang region for the years 1980, 1990, 2000 and 2010*

Table 1. *Temporal patterns of LULC changes of the Su-Xi-Chang region*

LULC type	1980		1990		2000		2010		Change (1980 – 2010)	
	Area (ha)	%	Area (ha)	%	Area (ha)	%	Area (ha)	%	Total	%
Farmland	977,687	56.04	922,383	52.87	864,338	49.54	647,986	37.14	-329,701	-33.71
Woodland	114,796	6.58	116,890	6.78	117,154	6.72	119,831	6.87	5,035	4.37
Grassland	1919	0.11	1745	0.11	1592	0.09	2203	0.13	284	14.81
Water body	514,839	29.51	516,757	29.61	499,309	28.62	485,055	27.8	-29,784	-5.79
Wetland	7153	0.41	5059	0.31	3672	0.21	3454	0.20	-3,699	-51.71
Construction land	126659	7.26	181,790	10.32	258,559	14.82	486,095	27.86	359,436	284.81
Total	1,744,624	100	1,744,624	100	1,744,624	100	1,744,624	100	-	-

Analysis of temporal LULC changes

The general tendency of change in LULC during the thirty-year period is indicated in *Table 1*. On the basis of the land use transition matrix, the comprehensive dynamics among different LULC types is shown in *Tables 2, 3 and 4*. With 56% coverage of the study area, farmland was the dominant cover type in 1980, followed by water body

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(30%). In the same period, construction land covered only 7% of the studied landscape (*Table 1*). In 1990, farmland decreased to 53% and water body was slightly balanced (30%), and both are the dominant LULC types. However, construction land increased by 3.2% of the preceding period, covering more than 10% of the total study area during this period (*Table 1*).

From 1980 to 1990, construction land, woodland, and water body enlarged by 3.4%, 0.2%, and 4%, respectively. In contrast, farmland, grassland, and wetland decreased by 1.3%, 9.6%, and 39%, respectively (*Table 2*). In this period, farmland was the main contributor to an increase in the areas of woodland, water body, and construction land by allowing for 27%, 48%, and 79% of the increase in their total area, respectively (*Table 2*). In this period, the grain for green development policy, expansion of artificial ponds for fish production, and urban expansion were the main reasons for the large amount of farmland conversion.

Table 2. *LULC conversion matrix from 1980 to 1990 (ha)*

								Change 1990	
Land use type	FL	WOL	GL	WB	WL	CL	Sum 1990	Total	%
Farmland (FL)	922,011	106	10	110	94	263	922,383	-55,304	-5.71
Woodland (WOL)	591	114,656	204	2	6	2	116,890	2,094	1.79
Grassland (GL)	396	13	1648	9	12	1	1745	-174	-9.01
Water body (WB)	9386	10	52	514,449	2830	3	516,757	1918	0.39
Wetland (WL)	1098	0	4	154	4181	13	5059	-2,094	-29.31
Construction land (CL)	44205	11	1	15	30	126477	181,790	5,513	4.35
Sum 1980	977,687	114,796	1919	514,839	7153	126659	1,744,624	-	-

From 1990 to 2000 farmland, grassland, and wetland continued decreasing by more than 6%, 8%, and 27% respectively. In addition to these three LULC categories water bodies started to decrease with more than 3% of its total area of the base year in the same period; however, woodland, and construction land expanded by 0.3%, and 42%, respectively (*Table 3*). In this period, the diminishing of farmland contributed 100%, and 5% to the expansion of construction land, and woodland, respectively (*Table 3*). In the period between 2000 and 2010, a comparable LULC change pattern was found, in which construction land continued to increase at an alarming rate, and was followed by grassland and woodland. The decreasing farmland area caused an increase in the areas of construction land, woodland, and grassland by 76%, 93%, and 100%, respectively (*Table 4*). In addition, a reduction in wetland area contributed 4% toward the expansion of construction land in the same period (*Table 4*). Thus, as lands supporting construction land expansion became scarce, wetlands the target for construction development. Our result supports the findings of Wang et al. (2011), who have found that the expansion of construction land in the West Songneu Plain of China led to the shrinking and fragmentation of swamplands. Similarly, Yuan Zhang (2010) has described the same phenomenon in the Yinchuan Plain of China.

Considering the LULC conversion matrix from 1980 to 2010, there was considerable growth in construction land over the other LULC types; remarkably, this growth far exceeded the amount of land converted from construction land to other LULC types.

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The results (*Tables 2, 3 and 4*) showed that over the study period, the loss of farmland strongly contributed to the newly emerging construction land, and was followed by wetland and water bodies, respectively. Thus, driven by robust socioeconomic factors, the ongoing growth in construction land was responsible for a large decline in the farmlands, wetlands and water bodies in the region.

Table 3. *LULC conversion matrix from 1990 to 2000 (ha)*

Land use type	FL	WOL	GL	WB	WL	CL	Sum 2000	Change 2000	
								Total (ha)	%
Farmland (FL)	828,938	198	0	2	1	1331	864,338	-58,045	−6.29
Woodland (WOL)	185	113,840	0	0	0	1	117,154	264	0.23
Grassland (GL)	6	58	1695	155	0	1	1592	-153	−8.77
Water body (WB)	8763	34	6	516,032	185	221	499,309	-17448	−3.38
Wetland (WL)	1	0	3	0	4856	110	3672	-1387	−27.42
Construction land (CL)	84,490	2760	41	620	17	180,126	258,559	76769	42.23
Sum 1990	922,383	116,890	1745	516,757	5059	181,790	1,744,624	-	-

Table 4. *LULC conversion matrix from 2000 to 2010 (ha)*

Land use type	FL	WOL	GL	WB	WL	CL	Sum 2010	Change 2010	
								Total (ha)	%
Farmland (FL)	646,970	703	23	96	18	6205	647,986	-216,352	−25.02
Woodland (WOL)	2593	111,390	16	50	0	109	119,831	2,677	2.27
Grassland (GL)	1124	2226	1274	58	1	155	2203	611	38.38
Water body (WB)	33,412	457	185	487,479	67	2456	485,055	-14,254	−2.85
Wetland (WL)	186	35	0	1498	3492	554	3454	-218	−5.93
Construction land (CL)	179,952	2343	94	10,128	94	249,044	486,095	227,536	88.01
Sum 2000	864,338	117,154	1592	499,309	3672	258,559	1,744,624	-	−

Analysis of landscape metrics

The overall trends of LULC fragmentation at both class (*Table 5*) and landscape (*Fig. 3*) levels were considered. The detailed descriptions of landscape metrics used in this study are given in Appendix B. As shown in *Table 5*, the landscape change for farmland and wetland at the class level indicated a consistent increase in the number of patches (NP) that enhanced the corresponding patch density during the study period. For farmland, NP increased from 357 in 1980 to 383 in 1990 and to 539 and 812 in 2000 and 2010, respectively, thus indicating that the spatial heterogeneity of this class increased with increasing disturbance over time. A similar pattern was found in the wetland category, as evidenced by increased patch density (PD) (*Table 5*). This result suggested that in recent decades, increasing human pressure has led to the fragmentation of both farmland and wetland LULC categories. The largest patch index (LPI) for both farmland and wetland showed a declining trend throughout the study period, owing to the intensification of construction land. For instance, the LPI for the wetland category

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decreased from 10.12% in 1980 to 2.04% in 2010. Likewise, the LPI for farmland decreased from 38.7% in 1980 to 27.5% in 2010 (*Table 5*). Furthermore, interspersion and juxtaposition (IJI) indicated identical configurations for both LULC types, thus indicating that the patch types of these LULC categories were more scattered.

Apart from farmland and wetland, and except for construction land, the rest of the LULC types showed irregular stability with variable values. However, as observed in the LULC change analysis (*Tables 2, 3, 4; and Fig. 2*), construction land has rapidly replaced farmland in the Su-Xi-Chang region. The NP for construction land was 10,510 in 1980, decreased to 10,252 in 1990 and increased to 10,835 in 2000, but in 2010, markedly decreased to 7641. The rapid urban development between 1990 and 2000 might have resulted in the large NP in 2000. The PD values also showed that the construction land became denser over time, a result also confirmed by its IJI values, which kept increasing, thus indicating that the construction land category has a continuous and clumped pattern.

Table 5. *Landscape metrics changes according to patch class level in the Su-Xi-Chang region*

Class	Year	NP	PD	LSI	LPI	AWMPFD	IJI
Farmland	1980	357	0.0205	69.0205	38.7074	1.1	62.2521
	1990	383	0.022	75.9841	34.2572	1.02	61.1539
	2000	539	0.031	93.1932	30.2799	1.21	56.0708
	2010	812	0.0467	103.8536	27.4821	1.26	56.7936
Woodland	1980	609	0.0363	38.8551	1.4845	1.26	41.071
	1990	641	0.035	38.5286	1.4822	1.23	41.2144
	2000	631	0.0369	39.9257	1.4601	1.06	50.6993
	2010	640	0.0368	40.9147	11.4319	1.02	60.4878
Grassland	1980	141	0.009	19.184	0.0385	1.23	88.902
	1990	156	0.0081	18.8301	0.0215	1.03	89.8603
	2000	152	0.009	19.5546	0.0214	1.01	93.1942
	2010	157	0.0145	24.5787	10.0182	1.02	92.6445
Water body	1980	2447	0.1407	40.8143	17.835	1.25	41.1412
	1990	2201	0.1265	39.4735	16.1191	1.2	39.6078
	2000	2548	0.1465	42.6489	15.187	1.26	43.7562
	2010	2534	0.1457	47.0361	14.477	1.16	47.2235
Wetland	1980	219	0.0126	42.3869	10.1173	1.08	61.3066
	1990	225	0.0143	52.7915	8.054	1.05	61.0185
	2000	238	0.0145	68.0608	5.0451	1.14	62.1501
	2010	287	0.0152	75.2681	2.0428	1.23	70.0982
Construction land	1980	10510	0.6043	138.6259	10.3137	1.2	14.5744
	1990	10252	0.5895	135.9465	20.3498	1.26	14.7907
	2000	10835	0.623	132.7572	26.7967	1.06	20.3902
	2010	7641	0.4393	97.3106	33.3579	1.05	29.8143

NP = number of patches; PD = patch density; LSI = landscape shape index; LPI = largest patch index; AWMPFD = area-weighted mean patch fractal dimension; and IJI = interspersion and juxtaposition.

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To designate the characteristic features of the fragmentation of LULC at the landscape level and to evaluate the extent of human pressure causing changes on landscape structure, the NP, LPI, AWMPFD, IJI, Shannon's diversity index (SHDI), and contagion index (CONTAG) were computed by using FRAGSTATS, and the results are presented in *Fig. 3*. The opposite magnitudes between LPI and NP at the landscape level (*Fig. 3a, and b*) indicated high landscape fragmentation. Thus increasing human activities in the region have caused a dispersed landscape structure. In addition, the continuously increasing value of AWMPFD indicated clear fragmentation of LULC at the landscape level (*Fig. 3c*). A continuous decline in values of SHDI indicated that the landscape was unevenly distributed and subjected to less patch types. The SHDI decreased from 1.2608 in 1980 to 1.1382 in 1990, and from 1.0377 in 2000 to 1.0352 in 2010 (*Fig. 3d*), possibly as a result of urban landscape dominance. The CONTAG value was lower in 1990 than in 1980. However, the values again became higher after 2000, thus suggesting a more contiguous and homogenous pattern. The analysis showed that the intensification of human activities in the study area has caused landscape fragmentation, thereby leading to the loss of the function of a natural landscape.

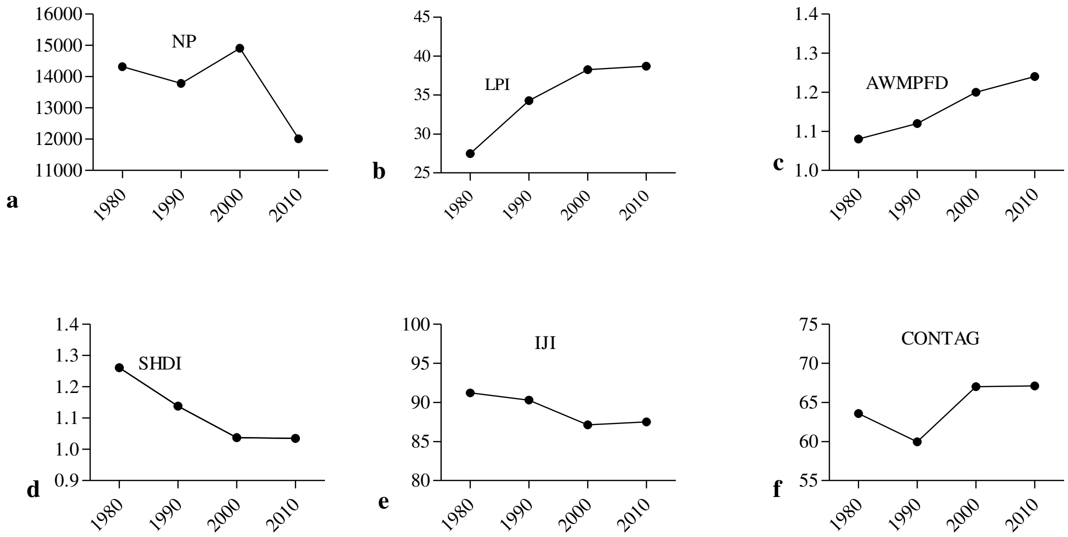

Figure 3. *Changes in number of patches (NP), largest patch index (LPI), area-weighted mean patch fractal dimension (AWMPFD), Shannon's (SHDI), interspersion and juxtaposition (IJI) and contagion index (CONTAG) at the landscape level*

Analysis of major socioeconomic drivers

Combinations of various socioeconomic factors have induced LULC changes in the Su-Xi-Chang region. *Table 6* depicts the relationships among these different socioeconomic variables and LULC changes. In addition, Appendix A shows the various socioeconomic variables considered in this study along with their detailed values over the past thirty-years. The overall results of our statistical analysis showed that over the past three decades, population growth and economic development, urbanization and subsequent construction land expansion, and rapid industrialization were the major drivers of LULC changes in the Su-Xi-Chang region.

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Population growth and economic development

The total population of the Su-Xi-Chang region was approximately 11 million in 1980, and suddenly increased to 13 million within a decade in 1990, with an annual growth rate of 8.6%. In 2000, the population rose to 13.5 million, and in 2010, it reached 14.5 million. In 2014, the population reached more than 15.2 million (NBSC, 2015). After population growth, LULC changes in the region became aggravated over time (*Table 1*). Different authors, particularly those in developing countries, have suggested that increased population growth causes LULC changes (Garedew et al., 2009; Kamusoko and Aniya, 2007).

Our results showed that the relationship between the growth in population and construction land was positively correlated (r = 0.631), and the values were significantly different (P<0.05). This result suggested that the increase in population also caused the increase in construction land expansion (*Table 6*), possibly because of an increase in settlement demand, for which large parts of agricultural lands were changed to residential areas, both in rural and urban zones. This possibility was confirmed by the inverse relationship between population growth and agricultural land (*Table 6*). Furthermore, the negative correlation between these two factors may suggest the importance of population growth in the LULC changes occurring in the region. This result is consistent with findings from Priess et al. (2007) in Indonesia and Kidane et al. (2012) in Ethiopia, which have shown that increases in population growth lead to agricultural land loss. Because Su-Xi-Chang is a potential region for agricultural production, a large part of this region is still in the agricultural sector. To meet the demands of the growing population by producing high production from limited land resources, large areas of agricultural land were converted into alternative productive land use practices, such as fishponds for fish farming, as evidenced by the strong positive relationship between the growth of population and increase in aquatic products (*Table 6; Fig. 4*).

Table 6. *Correlation coefficients between different socioeconomic variables*

	Population	Industrial output	Agricultural production	Aquatic products	GDP	Agricultural land	Construction land
Population	1	.407*	-.307	.479**	.405*	-.447*	.631*
Industrial output		1	-.882**	.715**	.999**	-.979**	.976**
Agricultural production			1	-.704**	-.867**	.880**	-.905**
Aquatic products				1	.706**	-.826**	.815**
GDP					1	-.973**	.971**
Agricultural land						1	-.982**
Construction land							1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

In addition to population growth, economic development showed a positive correlation (r = 0.971) with construction land, and the relationship was highly significant (P<0.01) (*Table 6*). Thus, an increase in economic development also caused an increase in

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construction land expansion. This result is in line with findings from Tang et al. (2014) in Harbin, Northeast China, which have indicated that economic development can cause LULC changes that significantly affect the functions of a natural landscape. Given the advantage of its location, being in a coastal area of the country, the economy of the Su-Xi-Chang region showed an upward trajectory (*Fig. 4a*), and currently, the region plays a vital role in the Chinese economy. This observation also accounted for the rapid expansion of urban and industrial lands that led to LULC change. Hence, our results showed that in general, the growth of the population and economic developments were the principal causal factors of changes in the LULC of the study area.

Industrialization

In the study period, industrialization in the Su-Xi-Chang region explosively increased after the rapid rise in the total industrial output value (*Fig. 4a*), which also caused major LULC changes. An all-around rapid industrialization and urbanization occurred in the region since 2000, owing to a higher consumption of land for industrialization than for urbanization. During this period, to support the manufacturing expansion, less attention was given to agricultural land. As a result, urban associated industrialization became an essential driving force of LULC changes, thereby decreasing the arable land category. Rural industrialization has been reported to contribute to construction land expansions in China (Lin and Ho, 2003). This phenomenon also occurred in the Su-Xi-Chang region, as evidenced by the strong positive correlation between construction land and the industrial output value ($r = 0.976$) with a significant difference of $P<0.01$.

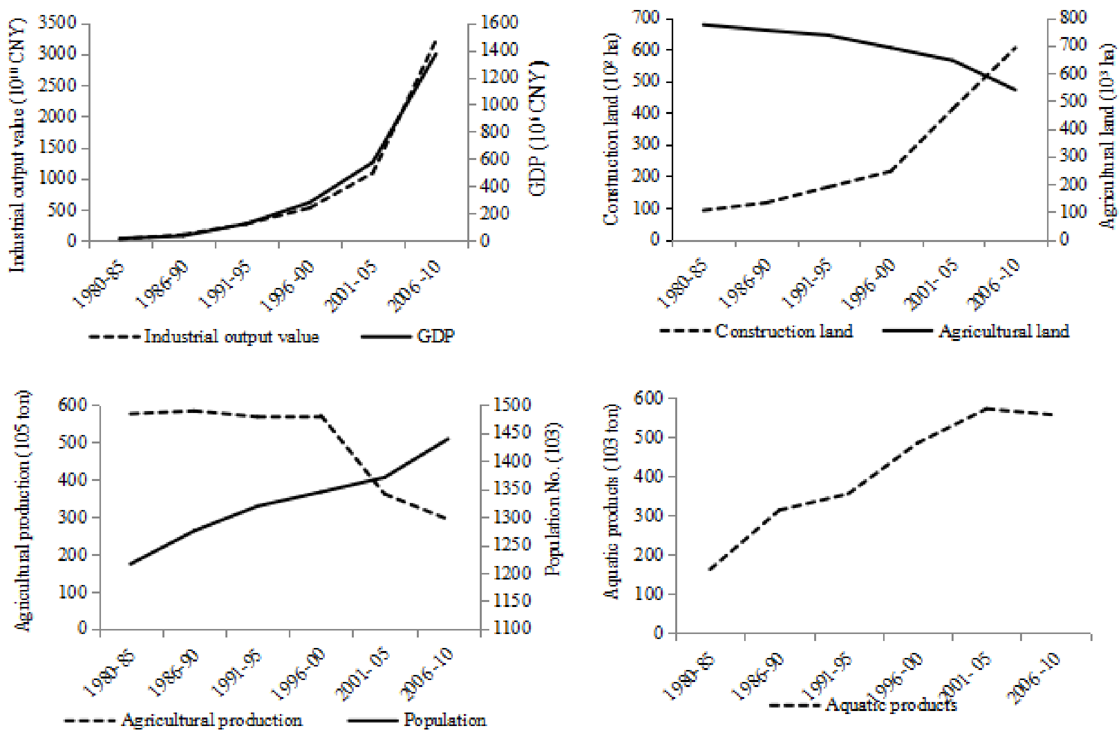

Figure 4. *Trends in different socioeconomic variables from 1980 to 2010 in the Su-Xi-Chang region*

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As indicated in *Table 6*, there was a strong positive correlation between aquatic products and the industrial output value (r = 0.715) and between GDP and the industrial output value (r = 0.999), both of which exhibited significant differences (P<0.01). These findings suggested that increases in both aquatic products and GDP contributed to the increase in industrial output. This finding may have been due to an increase in the row material supply from increased aquatic products, as well as to an increase in GDP, which enhanced the output values of industries. However, the industrial output value was negatively correlated with agricultural production (r = -0.882) and agricultural land (r = -0.979), and the relationships were highly significant (P<0.01), thus indicating the effect of industrialization from the massive conversion of agricultural land into market oriented land use. Put another way, the results indicated that industrialization has cursorily caused substantial loss of agricultural land, to the benefit of market oriented farming and non-agricultural development.

In addition, owing to economic prosperity, particularly by the middle of the 1980s, the region provided a model for the development of rural industries. This model also describes when the Township and Village Enterprises (TVEs) thrived after rapid growth of the rural economy in southern Jiangsu (Long et al., 2009). Since then, the growth of TVEs and the development of an export-oriented economy have fundamentally transformed the industrial pattern of the region. As a result, the total industrial output value markedly increased (*Fig. 4a*). These manifestations played roles in LULC changes, as evidenced by the strong inverse relationship between industrial output values and the loss in agricultural land (*Table 6*).

Urbanization and subsequent construction land expansion

Rapid urbanization was stimulated after the Chinese economic reform of the 1970s, particularly around the beginning of the 1990s, and urbanization was accelerated to a great extent as the centrally planned economy shifted to a socialist market. This shift led to construction land expansion (Xu, 2004; Liu et al., 2005) and subsequently to the loss of agricultural land in China (Kuang et al., 2016). In the Su-Xi-Chang region, the area of construction land, mainly owing to urbanization, increased from 126,659 ha in 1980 to 181,790 ha in 1990, with an annual growth rate of 4.35% (*Table 1*). In 2000, it attained a total area of 258,559 ha, and in 2010, it increased by 227,536 ha more than that in the former period, in which farmland contributed 76% of the amplification (*Table 4*). These changes were evidenced by the strong inverse relationship between construction land and agricultural land (*Table 6; Fig. 4b*). In the past three decades, notably, construction land increased by 359,436 ha, amounting to 284.81% of the area in 1980, during which it covered merely 7% of the total landscape, which rose to 27.86% in 2010 (*Table 1*).

The results showed that areas of 79%, 100%, and 76% enlargement in construction land from 1980 to1990, 1990 to 2000, and 2000 to 2010, respectively, were contributed by farmland (*Tables 2, 3, and 4*). As (Liu et al., 2008) have described, this type of occupation mostly occurred in the developed areas, especially in the main grain production zone of the coastal regions, thus leading to simultaneous decreases in both the area and production of agricultural land (*Fig. 4b and c*). Particularly, the decrease in production occurred because the productive lands occupied by construction land and crop cultivation were forced to be located in less productive areas. This scenario can be seen in *Table 6*, which shows the negative relationship between agricultural production

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and construction land (r = -0.905) as well as agricultural land and construction land (r = -0.982), with a highly significant correlation (P<0.01).

The occurrence of various development activities in the region also played vital roles in the construction land expansion, which was followed by a decrease in agricultural land (*Fig. 4b*). For instance, the construction of a special development zone, the ETDZ, in Kunshan City of the Su-Xi-Chang region originated in 1985 (Long et al., 2007). It was approximately 375 ha before it was chosen as one of the state development zones. After a decade, its planning area increased by more than 500%, and it attained a total area of 2000 ha. Correspondingly, the advent of TVEs to increase farmers' non-agricultural income by promoting the structural adjustment and institutional innovation made a vital contribution to the process of expanding construction land, thus resulting in conversion of large areas of agricultural land for rural village construction. Thus, urbanization and the subsequent expansion of construction land was generally one of the most important factors in LULC change in the studied landscape.

Conclusion

We examined the spatio-temporal dynamics of LULC changes, major socioeconomic drivers, and the relationship between changes in LULC and their drivers in the Su-Xi-Chang region over the course of 1980 to 2010. Significant changes in LULC were found in the region, whereby construction land dramatically increased largely at the expense of farmland. From 1980 to 1990, diminishing farmland was more important for the newly emerged construction land, as well as for the enlargement of water bodies and woodland, than the other cover types. In the same period, dwindling wetland contributed to construction land and water body increases next to farmland. Similar trends of conversion occurred in the periods from 1990 to 2000 and 2000 to 2010, except for variations in the spatial extent of changes among the different categories. In addition, changes in landscape patterns and composition exhibited increased the fragmentation of farmland and wetlands. However, the construction land category became aggregated and complex, thereby resulting in the high fragmentation of other LULC categories.

Population growth and economic development, urbanization and subsequent construction land expansion, and industrialization were found to be the major change drivers. The growth of population over time increased the demand for residential areas, and hence, large areas of farmland were converted for settlement construction. Furthermore, to achieve more economic benefits from their farmland, farmers practiced profit oriented farming systems, particularly by converting their farmland into artificial ponds for fish production. Similarly, the rapid industrialization consumed large areas of farmland. In general, the marked expansion of construction land is mainly accountable for the 33% decrease in farmland in the past three decades.

Given the current trends of socioeconomic drivers in the Su-Xi-Chang region, increasing pressure on LULC is causing an alarming change in farmland, water bodies, and wetland. The change in farmland involves decreases in both area and production and may impair the ecological functions that support the human dominated environment and result in a decline in food production. Therefore, policies to achieve long term sustainable development must address the effects of these drivers on LULC changes. Moreover, further research along these lines should be encouraged because additional studies will be helpful for the decision makers engaged in planning activities at various levels in the region.

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APPENDIX

Appendix A. *The various annual series of socioeconomic data of the Su-Xi-Chang region over the past 30 years used for this study*

Year	Population ($\times 10^3$)	Ind. Output (10^9 CNY)	Agri. Pro ($\times 10^5$ ton)	Aqu. Pro ($\times 10^3$ ton)	GDP ($\times 10^6$ CNY)	Agri. Land ($\times 10^3$ ha)	Cons. Land ($\times 10^2$ ha)
1980	1191.44	210	540.39	120.66	9.9	781.36	80.9
1981	1204.55	230	487.42	125.586	10.61	779.85	80.5
1982	1217.34	240	610.62	152.132	11.44	777.6	90.6
1983	1222.89	280	604.22	151.164	12.85	776.88	96
1984	1225.95	360	676.13	185.776	16.61	772.91	99.7
1985	1233.93	510	538.77	231.141	22.14	765.55	105.1
1986	1245.57	600	602.55	278.718	15.42	760.86	108.6
1987	1260.22	750	565.21	303.477	29.71	757.58	111.9
1988	1275.2	990	581.32	318.259	38.1	753.8	117

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1989	1290.68	1110	570.34	322.999	40.86	752.77	120.5
1990	1303.58	1230	601.95	337.012	45.75	751.26	124.2
1991	1307.97	1330	576.96	324.575	52.21	750.16	137.9
1992	1312.36	1440	551.97	312.139	80.81	749.06	154.1
1993	1319.36	2410	591.95	348.315	119.02	737.88	173.2
1994	1325.14	3570	567.72	372.112	164.04	729.06	180.4
1995	1331.19	4740	552.64	412.938	202.85	720.83	188.5
1996	1335.75	4720	578.81	452.861	229.51	712.54	201.8
1997	1342.48	4870	603.88	464.161	255.05	691.36	207.8
1998	1346.51	5010	608.56	476.479	279.29	688.08	215.5
1999	1348.31	5460	542.7	501.561	301.77	685.19	222.1
2000	1349.34	6070	525.57	521.389	331.79	684.36	231.3
2001	1354.26	7090	457.19	558.672	376.18	681.59	330.9
2002	1357.95	7750	391.9	577.017	437.51	673.68	378.1
2003	1365.68	9490	365.51	588.776	553.64	652.28	416.4
2004	1379.73	12,520	288.01	575.635	680.28	638.65	454.6
2005	1395.01	17,380	305.06	553.112	825.52	587.86	487.5
2006	1411.78	21,600	283.81	567.64	979.66	575.32	507.1
2007	1428.55	27,230	307.16	562.868	1164.33	546.83	531
2008	1443.55	33,960	261.91	550.901	1380.5	536.62	633.3
2009	1452.69	37,590	306.51	557.026	1525.19	531.74	661
2010	1458.76	40,530	307.63	535.986	1806.71	513.35	696.9

NB: Ind. output = Industrial output value; Agri. Pro = Agricultural production; Aqu.pro = Aquatic products; Agri. Land = Agricultural land; Cons. Land = Construction land (in this particular case it stands for residential area).
(**Sources**; Statistical year books and different surveys deployed in different time by the governmental offices of the region).

Appendix B. *Landscape metrics selected for this study and their description*

Acronym	Name (units)	Description	Justification
NP	Number of patches	Total number of patches in the landscape	Fragmentation
PD	Patch density (per 100 ha)	Number of patches per unit area	Fragmentation
LSI	Landscape shape index	The landscape boundary and total edge within the landscape divided by the total area, adjusted by a constant for a square standard	Aggregation

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LPI	Largest patch index (%)	Area of the largest patch in each class, represents percent of the total landscape area	Dominance
SHDI	Shannon’s diversity index	SHDI equals minus the sum, measure of diversity. Approaches 0 when there is no diversity, increases with number of patch types.	Diversity
IJI	Interspersion and juxtaposition index (%)	Intermixing of patches of different types, based on patch adjacencies. Increases to 100 as the patch type becomes increasingly interspersed with other patch type.	Fragmentation
CONTAG	0 < CONTAG ≤ 100 Contagion index (%)	Approaches 0 when the patch types are maximally disaggregated and interspersed. Approaches 100 when the landscape consists of a single patch.	Fragmentation
AWMPFD	Area-weighted mean patch fractal dimension	Shape complexity weighted by the area of patches	Fragmentation

Adapted from McGarigal et al. (2002).

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PLANT GROWTH REGULATORS IN SEED COATING AGENT AFFECT SEED GERMINATION AND SEEDLING GROWTH OF SWEET CORN

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Abstract. To evaluate the potential effectiveness of plant growth regulators in improve seed germination and seedling vigor when applied during seed coating in sweet corn. 6-benzylaminopurine (6-BA), 1-naphthalene acetic acid (NAA), brassinolide and gibberellic acid (GA_3) were added as seed coating agent, and seed germination, antioxidant capacity and seedling vigor of sweet corn were investigated. The results showed compared with the use of coating agent alone, plant growth regulators improved seed vigor and germination, especially GA_3 (200 and 250 mg L^{-1}) and 6-BA (20 and 40 mg L^{-1}) were added. Furthermore, 200 mg L^{-1} GA_3 treatment improved seed germination and antioxidant capacity and resulted in sweet corn seedlings with a better appearance. The results observed indicated plant growth regulators might be valuable agents in sweet corn seed coating.
Keywords: *sweet corn, gibberellic acid (GA_3), 6-benzylaminopurine(6-BA), seed coating agent, seed vigor*

Introduction

Sweet corn hybrids carrying the *shrunken 2 (sh2)* gene, or supersweets, are extensively planted worldwide (Parera, 1990). Laughnan (1953) studied the effects of the *sh2* gene on carbohydrate reserves in maize endosperm and found that *sh2* endosperm stored less starch than normal types and possessed approximately 10-fold higher levels of total soluble sugars, with most of this increase being attributable to sucrose. Because of the higher levels of sugar in endosperm and a high sugar retention after harvest, sweet corn with the *sh2* gene provides superior quality and consumer appeal and permits longer transport and processing times (Duan, 1997).

Despite these superior features, the commercial acceptance and widespread use of *sh2* hybrids has been limited by poor seed quality. This poor seed vigor has been attributed to various factors, such as an insufficient nutrient supply during seed germination due to the low starch concentration. In addition, the higher imbibition rate of *sh2* kernels that leads to severe solute leakage increases susceptibility to physical damage and seed- and soil-borne diseases (Garwood et al., 1976; Styer et al., 1983). As a result, the yield and profitability of sweet corn possessing the *sh2* gene is hindered by poor seed vigor, as reflected in decreased emergence, poor seedling vigor, and erratic

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stand uniformity (Tracy, 1989; Parera et al., 1991).

Although various seed treatments, including fungicide treatment, pre-sowing hydration and bio-priming, are effective for improving sweet corn seed germination and seedling growth (Bennett et al., 1987; Tracy, 1989; Callan et al., 1990; Wilson et al., 1992; Hartz et al., 1995; Zhang et al., 2007), these methods have not been used on a commercial scale. As *sh2* hybrid corn is becoming a major commercial sweet corn genotype, seed coating technology that enhances seed value and promotes the mechanization of the planting process has attracted increasing attention. According to one study, the performance and physical properties of rice seeds are improved by coating them with liquid-based polymeric adhesives (Zeng et al., 2009). In addition, the oxygen provided to rice seeds planted under anoxic or near-anoxic soil conditions is increased when seeds are coated with peroxide compounds (Baker et al., 1987; Sono et al., 1991). Furthermore, germination and survival rates of seeds under adverse environmental conditions are promoted by coating with polymers incorporating pesticides (Taylor et al., 2001; Manjunatha et al., 2008). In sweet corn, however, seed germination is inhibited by coating with either polymers alone or polymers incorporating pesticides (Ikekawa et al., 1991; Lan et al., 2008). Plant growth regulators are active ingredients in coating agents, but their effects on seed coating have rarely been investigated. A In this study, we therefore conducted lab experiments to investigate the effects of plant growth regulators 6-benzylaminopurine (6-BA), 1-naphthalene acetic acid (NAA), brassinolide (BR) and gibberellic acid (GA_3) in seed coating agents on seed germination and seedling growth of sweet corn.

Materials and Methods

The study was conducted at the Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangdong, China, during the summer of 2013.

Preparation of seed coating agent

Seed coating agent containing no active ingredients was provided by Incotec (Beijing, China). A GA_3 stock solution was prepared by dissolving 0.125 g of GA_3 in 1 mL absolute ethyl alcohol, followed by dilution with water to 250 mL and pH adjustment to 6.8–7.0. For use in subsequent experiments, this stock solution was diluted with water to give concentrations of 50, 100, 150, 200 and 250 mg L^{-1}. NAA (10, 20, 40, 60 and 80 mg L^{-1}), 6-BA (20, 40, 60, 80 and 100 mg L^{-1}) and BR (5, 10, 15, 20 and 25 mg L^{-1}) solutions were prepared in a similar fashion. The different plant growth regulator solutions were mixed with the Incotec seed coating agent in a 1:4 (v/w) ratio.

Seed coating treatments

The supersweet corn cultivar ‘Zhengtian 68’ bred at the Crops Research Institute, was used as seed material. All seeds were film-coated by hand. Seeds and seed coating agents with plant growth regulators were poured into a large plastic bag in a 1:50 (w/w) ratio. The bag was tightly closed and shaken to ensure even distribution of seed coating agents on seeds. The coated seeds were air-dried at room temperature for 2 h and then stored at 4 °C for 1 month.

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Germination testing

Samples comprising 150 seeds per treatment were placed on three Petri dishes (50 seeds per dish) and incubated in a growth chamber under controlled conditions (25–28 °C, 12-h photoperiod and 80–85 % relative humidity). Three replications were used for each treatment. Two controls were also set up: uncoated seeds (CK1) and seeds coated with coating agent without plant growth regulators (CK2). The number of germinated seeds was recorded daily for 1 week, and root and shoot lengths were measured 7 days after sowing. Germination potential, germination rate, germination index and vigor index were calculated according to Crop Seed Inspection Procedures of the National Standard of the People's Republic of China (GB/T 3543.1-1995) as follows: Germination rate (%) = (number of germinated seeds 7 days after sowing / total seed number) × 100 %; Germination potential (%) = (number of germinated seeds 3 days after sowing / total seed number) × 100 %; Germination index (GI) = $\sum(Gt/Dt)$, and Vigor index = GI × S, where Gt is the number of germinated seeds on day *t*, Dt is the number of germination days, and S is seedling weight (in g).

Plant sampling and enzyme activity measurements

Treated and control seeds were sown in 10-cm diameter plastic containers and germinated in a growth chamber under controlled conditions (25–28 °C, 12-h photoperiod and 80–85 % relative humidity). Ten days after germination, fresh leaves from each treatment were sampled in liquid nitrogen, ground into a paste in an ice bath with 4 mL of 0.05 M phosphate buffer (pH = 7.8), transferred to a 10-mL centrifuge tube and centrifuged at 7,000 × *g* for 20 min. The resulting supernatant fluid was stored at −80 °C for measurement of superoxide dismutase (SOD), peroxidase (POD), malondialdehyde (MDA) and catalase (CAT) enzyme activities.

SOD activity was assayed by measuring the ability of the solution to inhibit the photochemical reduction of nitroblue tetrazolium following the method of Stewart et al. (1980). CAT activity was measured as the decline in absorbance at 240 nm due to the decrease of extinction of H_2O_2 using the method of (PATRA et al. 1978). POD activity was determined using the method of (AMAKO et al. 1994). In particular, the absorbance change of brown guaiacol at 460 nm was recorded to calculate POD activity, with one unit of POD enzyme activity defined as the amount of enzyme causing an increase of 1.0 in absorbance in 1 min due to guaiacol oxidation. The level of leaf senescence was determined by measuring the amount of MDA following the method of (Vos et al., 1991). Absorbance was recorded at 532 nm, with measurements corrected for non-specific turbidity by subtracting the absorbance at 600 nm. MDA concentration was determined on the basis of the extinction coefficient.

Statistical analysis

Data were analyzed using SPSS 19.0. Data were presented as mean ± SEM. One-way ANOVA followed by Tukey test was used to compare mean values of the groups.

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Results

Seed germination

After storage at 4 °C for 1 month, seeds from all coating treatments, except for 200 and 250 mg L^{-1} GA_3 and 10 mg L^{-1} NAA, displayed significantly lower germination rates and germination indexes compared with CK1. The maximum germination potential was observed from the 200 mg L^{-1} GA_3 seed coating treatment, with no significant differences in germination potential found between the control and any NAA and 6-BA treatments, BR treatments except for 10 mg L^{-1} and the 200 and 250 mg L^{-1} GA_3 treatments. Coating treatments with 200 and 250 mg L^{-1} GA_3 gave rise to statistically significant increases in the vigor index compared with CK1, while 150 mg L^{-1} GA_3 and 20 and 40 mg L^{-1} 6-BA produced no significant differences in vigor index compared with CK1. The other treatments, namely 50 and 100 mg L^{-1} GA_3, 6-BA higher than 60 mg L^{-1}, and all levels of NAA and BR, resulted in a significantly lower vigor index compared with that of CK1.

Compared with CK1, all germination indexes—germination rate, germination potential, germination index and vigor index—were significantly decreased by the CK2 treatment. In particular, 150, 200 and 250 mg L^{-1} GA_3 and 10 mg L^{-1} NAA coating treatments caused statistically significant increases in germination rate relative to those from CK2 treatments, with the 100 mg L^{-1} 6-BA treatment producing seeds with the lowest germination rate among those from all coating treatments. The CK2 treatment was responsible for the lowest germination potential, and a significant increase was observed from all coating treatments except for 50 and 100 mg L^{-1} GA_3. Compared with CK2, the germination index was significantly increased by coating treatments of 200 and 250 mg L^{-1} GA_3, 20, 40 and 60 mg L^{-1} 6-BA and 10 mg L^{-1} NAA; no significant differences were observed as a result of the other coating treatments. Seed coating treatments of 200 and 250 mg L^{-1} GA_3 and 20 and 40 mg L^{-1} 6-BA produced seeds with statistically significantly higher vigor indexes compared with those from CK2 treatments. Higher levels of NAA (>10 mg L^{-1}) and 6-BA (>60 mg L^{-1}) and low levels of GA_3 (50 mg L^{-1}) significantly decreased the vigor index relative to CK2. No significant difference was observed after treatment at any BR level or with 10 mg L^{-1} NAA or 100 or 150 mg L^{-1} GA_3 (*Table 1*).

After storage for 2 months, germination indexes of sweet corn seeds were all decreased compared with values recorded after 1-month storage. The effects of seed coating treatments with different plant growth regulators all showed the same trends after storage for 2 months as those observed after 1 month (*Appendix 1*).

Table 1. *Effect of plant growth regulators in seed coating agent on seed germination of sweet corn*

Treatment (mg L^{-1})	Germination rate (%)	Germination potential (%)	Germination index	Vigor index
CK1	93.33±0.88a	59.67±0.33abc	30.39±0.24a	6.38±0.05B
CK2	78.00±1.73def	42.33±1.76i	24.76±0.70ghi	5.45±0.15CDE
GA_3-50	84.00±2.89bcde	47.33±0.88hi	25.29±0.41fgh	4.55±0.07GH
GA_3-100	85.00±2.08bcd	48.00±1.73ghi	25.66±1.00efgh	5.64±0.22CD
GA_3-150	85.67±2.03bc	51.67±1.67efgh	26.73±0.76cdefg	5.88±0.17BC
GA_3-200	89.67±0.33ab	61.67±1.20a	29.12±0.50ab	7.28±0.12A
GA_3-250	87.67±0.67ab	57.00±0.58abcde	28.71±0.25abcd	7.46±0.06A
6BA-20	84.00±2.52bcde	60.67±1.20ab	27.72±0.57bcde	6.37±0.13B
6BA-40	82.67±0.88bcde	60.33±0.88abc	27.76±0.41bcde	6.38±0.09B

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6BA-60	82.33±2.03bcde	58.33±1.45abcd	27.36±0.50bcdef	4.93±0.09EFG
6BA-80	72.00±1.00fg	56.00±2.08abcde	24.82±0.34ghi	4.22±0.06H
6BA-100	67.33±3.53g	49.33±1.67fgh	22.60±0.77i	3.62±0.12I
NAA-10	86.67±2.33ab	60.33±1.20abc	28.87±0.48abc	5.49±0.09CDE
NAA-20	79.00±2.00cdef	60.00±0.58abc	26.61±0.61defg	4.79±0.11FG
NAA-40	77.33±4.18ef	58.33±2.19abcd	26.46±1.35defg	4.23±0.22H
NAA-60	74.00±1.53fg	55.67±1.86abcde	25.32±0.67fgh	4.05±0.11HI
NAA-80	74.00±2.52fg	54.67±1.33bcdef	25.18±0.82fgh	3.52±0.11I
BR-5	77.33±3.48ef	57.33±2.91abcde	25.17±0.83fgh	5.54±0.18CD
BR-10	77.67±0.33def	52.33±4.33defgh	24.47±0.78ghi	5.14±0.16DEF
BR-15	71.67±3.28fg	56.00±2.08abcde	23.96±1.04hi	5.27±0.23DEF
BR-20	76.67±1.67ef	54.00±3.61cdefg	24.57±0.60ghi	5.65±0.14CD
BR-25	77.67±0.33def	55.00±1.15bcdef	24.88±0.39gh	5.22±0.08DEF

Note: different lowercase and uppercase letters are used to indicate values that are significantly different at $p < 0.05$ and $p < 0.01$, respectively

Root and shoot lengths

Compared with CK1, all treatments caused significantly lower root lengths. The 100–250 mg L^{-1} GA_3 treatments significantly increased shoot lengths, while no significant difference in shoot length was observed from 50 mg L^{-1} GA_3 or 20 or 40 mg L^{-1} 6-BA. Higher levels of 6-BA and BR, as well as all levels of NAA, had a significant inhibitory effect on shoot length (*Table 2*).

Table 2. *Effect of plant growth regulators in seed coating agent on root and shoot lengths of sweet corn seedlings*

Treatment	**Root length（cm）**	**Shoot length（cm）**
CK1	18.44±0.08A	8.88±0.05DE
CK2	16.86±0.12EFG	8.10±0.08FG
GA_3-50	16.74±0.16FG	8.75±0.03E
GA_3-100	17.15±0.03DEF	9.86±0.01C
GA_3-150	17.68±0.17BC	10.55±0.07B
GA_3-200	17.98±0.12B	11.56±0.04A
GA_3-250	17.40±0.05CD	10.76±0.06B
6BA-20	16.10±0.14I	9.03±0.08D
6BA-40	15.24±0.10J	8.70±0.03E
6BA-60	13.25±0.05L	7.68±0.03IJ
6BA-80	10.99±0.08M	7.02±0.07L
6BA-100	10.26±0.11N	6.70±0.04M
NAA-10	17.29±0.04CDE	7.90±0.09GH
NAA-20	16.94±0.03EFG	7.56±0.06JK
NAA-40	16.25±0.04HI	7.44±0.05K
NAA-60	14.62±0.17K	7.36±0.06K
NAA-80	13.54±0.04L	7.11±0.06L
BR-5	16.63±0.03GH	8.93±0.08DE
BR-10	16.15±0.17I	8.31±0.07F
BR-15	15.54±0.13J	8.24±0.01F
BR-20	14.61±0.03K	8.13±0.04F
BR-25	13.46±0.18L	7.83±0.04HI

Note: different uppercase letters are used to indicate values that are significantly different at $p < 0.01$

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Compared with CK2, treatments with 150, 200 or 250 mg L^{-1} GA_3 dramatically increased root lengths. No significant differences in root lengths were observed following treatment with coating agents containing 10 or 20 mg L^{-1} NAA, whereas the other treatments, namely, lower levels of GA_3 (50 and 100 mg L^{-1}), higher levels of NAA (40, 60 and 80 mg L^{-1}) and all levels of BR and 6-BA, significantly inhibited root lengths (*Table 2*). A significant enhancement in shoot length was produced by all GA3 treatments as well as 20 and 40 mg L^{-1} 6-BA and 5 mg L^{-1} BR treatments. No significant differences in shoot lengths were observed following 10, 15 or 20 mg L^{-1} BR treatments, while a significant decrease was observed after treatments involving 60, 80 or 100 mg L^{-1} 6-BA, 25 mg L^{-1} BR and all NAA levels (*Table 2*).

According to above data, coating treatments with 200 mg L^{-1} GA3 gave rise to statistically significant increases in the vigor index and 100 mg L^{-1} GA3resulted in a significantly lower vigor index compared with that of CK1. Sweet corn seedlings at 7 days after sowing from seeds subjected to CK1, CK2, 200 mg L^{-1} GA3 and 100 mg L^{-1} 6-BA coating treatments are presented in *Fig. 1*. As is obvious from the figure, seedlings from the 200 mg L^{-1} GA3 treatment had an outstanding appearance that contrasted with those from 100 mg L^{-1} 6-BA.

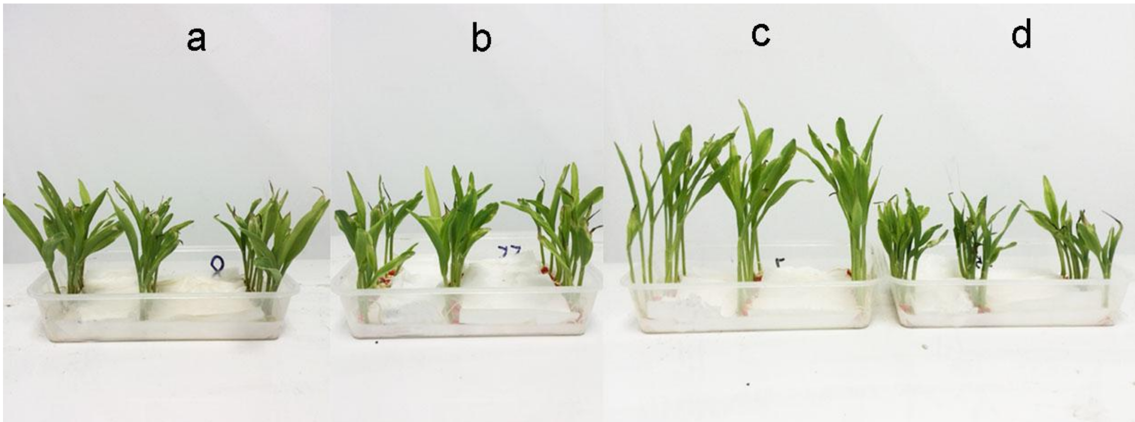

Figure 1. *Appearance of sweet corn seedlings at 7 days after sowing from seeds subjected to different seed coating treatments. a: uncoated seeds (CK1); b: no plant growth regulator in the coating agent (CK2); c: 200 mg L^{-1} gibberellic acid (GA_3); d: 100 mg L^{-1} 6-benzyladenine (6-BA)*

Physiological resistance

A pot experiment was conducted to investigate the physiological resistance of seedlings from selected treatments (CK1, CK2, 200 mg L^{-1} GA_3 and 100 mg L^{-1} 6-BA). Compared with CK1 and CK2, 200 mg L^{-1} GA_3 was respectively found to significantly increase SOD activities by 5.69 and 6.83 %, POD activities by 26.05 and 4.83 % and CAT activities by 18.98 and 14.67 % and to significantly decrease MDA content by 29.26 and 39.69 %. With respect to SOD, POD, and CAT activities and MDA content, the following trends were observed: SOD activity, 200 mg L^{-1} GA_3 > 100 mg L^{-1} 6-BA > CK1 > CK2; POD activity, 100 mg L^{-1} 6-BA > 200 mg L^{-1} GA_3 > CK2 > CK1; CAT activity, 200 mg L^{-1} GA_3 > 100 mg L^{-1} 6-BA > CK2 > CK1; and MDA content, CK2 > CK1 > 100 mg L^{-1} 6-BA > 200 mg L^{-1} GA_3.

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Discussion

The seed coating process involves application of pesticides, fertilizers, oxygen agents or growth regulators to seeds to resist diseases and pests, and to promote seed germination and seedling growth (Taylor et al., 2001; Zhang et al., 2007). Previous studies have shown seed coating technology to be an effective approach for improving seed germination and seedling growth of crop plants (Taylor et al., 2001). In rice, seed coating improves the performance and physical properties of seeds, especially under adverse environmental conditions (Baker et al., 1987; Sono et al., 1991; Tylor et al., 1998; Manjunatha et al., 2008; Zeng et al., 2009). Seed coating with salicylic acid, paclobutrazol or humic acid has a positive effect on seed germination and seedling growth in maize (Lan et al., 2008; Wang et al., 2010; Zhu et al., 2013). Boschi et al. (2014) reported the effect of 6-BA on the germination and growth of seeds of *Ginkgo biloba* and suggested that seed immersion with 2.5 ppm of 6-BA performed.

Sweet corn, with its naturally poor seed vigor, differs from other maize types (Harris et al., 1989). A key agricultural objective to increase sweet corn yields is achievement of rapid, uniform germination and seedling emergence (Rajjou et al., 2012). However, we found that a coating treatment lacking plant growth regulators inhibited seed germination and vigor (*Table 1*), which was consistent with previous studies (Ikekawa et al., 1991; Lan et al., 2008). This inhibition may be due to physical damage caused by seed coating.

Experimental coating with different concentrations of 6-BA and NAA showed that low concentrations of these plant growth regulators promoted seed germination, whereas high concentrations inhibited it (*Table 1*). Suitable concentrations of 6-BA (20–40 mg L^{-1}) and NAA (10–20 mg L^{-1}) were beneficial for rapid, uniform germination and seedling emergence. BR is an important phytohormone that plays an important role in various aspects of plant growth and development, including seed germination (Wilen et al. 1995; Dhaubhadel et al. 1999; Khripach et al. 2000; Miransari et al. 2014). Although seed immersion with BR has been found to promote seed germination in maize (Zou, 2002), we observed no obviously positive effect in coating treatments incorporating BR in the coating agent.

We found that coating using coating agent alone inhibited seed germination, as reflected by decreased germination rate, germination potential, germination index and vigor index. In contrast, coating treatments incorporating 200 and 250 mg L^{-1} GA_3 maintained a high germination rate, germination index and germination potential similar to CK1 and significantly increased vigor index and shoot length compared with CK1 (Tables 1 and 2). These positive effects may be due to the roles of GA in breaking dormancy and promoting seed germination and stem elongation (Gurdiola, 1996). In addition, 200 mg L^{-1} GA_3 significantly increased SOD, POD and CAT activities and decreased the accumulation of MDA content compared with CKs (*Figs. 1* and *2*). This result implies that GA_3 coating treatments improve seed germination and seedling physiological resistance by increasing SOD, POD and CAT activities in seedlings and by reducing membrane damage during the coating process.

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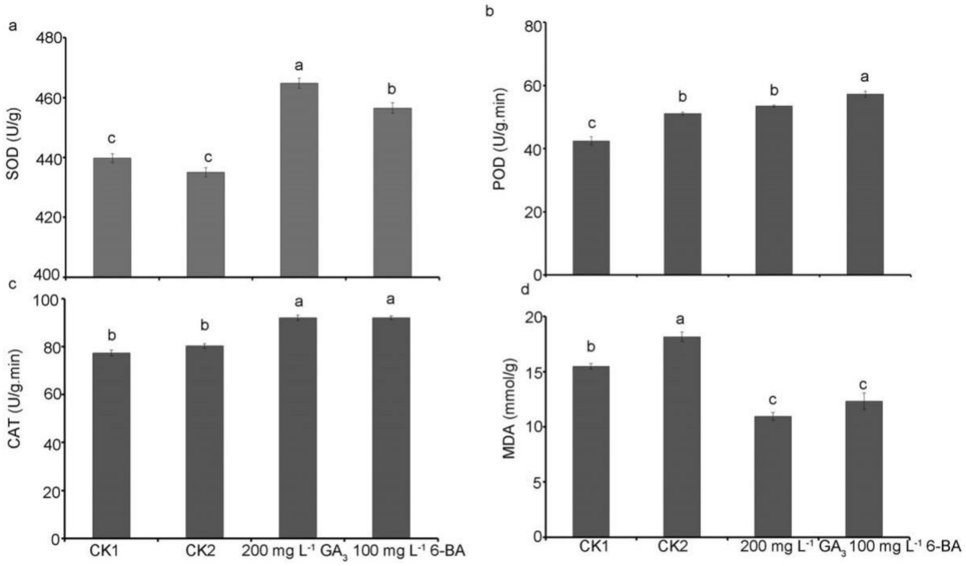

Figure 2. *Superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) contents of sweet corn seedlings at 10 days after sowing. Different lowercase letters indicate values that are significantly different at p < 0.05*

Conclusion

In this study, we found seed coating using coating agent alone inhibited seed germination, whereas, coating with suitable concentrations of 6-BA (20–40 mg L^{-1}), GA_3 (200 mg L^{-1}) and NAA (10–20 mg L^{-1}) were beneficial for rapid, uniform germination and seedling emergence in sweet corn. Which implied it is effective approach to improve seed germination through developing seed coating incorporated with suitable concentrations plant regulators (6-BA, GA_3, NAA) in sweet corn.

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APPENDIX

Appendix 1. *Effect of plant growth regulators in seed coating agent on seed germination of sweet corn after storage of seeds for 2 months*

Treatment （mg L^{-1}）	Germination rate （%）	Germination potential （%）	Germination index	Vigor index
CK1	91.00±1.00A	50.67±0.67A	27.45±0.19A	5.49±0.73A
CK2	56.67±0.88DEF	28.33±0.67EFGH	16.71±0.34FGH	3.18±0.09E
GA_3-50	57.00±1.56EDF	34.67±0.33CD	17.57±0.51DEF	3.34±0.09DE
GA_3-100	58.00±1.15DE	36.00±1.00C	18.51±0.33CD	3.52±0.11E
GA_3-150	62.33±0.33BC	37.67±0.88BC	19.23±0.19C	3.85±0.11C
GA_3-200	65.00±1.15B	40.67±0.67B	20.87±0.19B	4.17±0.06B
GA_3-250	62.23±0.33BC	37.00±1.00BC	19.32±0.26C	4.06±0.25BC
6BA-20	55.00±1.15EFG	29.67±1.33EFG	16.54±0.44FGH	3.31±0.14DE
6BA-40	53.67±0.67FGH	27.00±0.58FGH	16.08±0.13GHI	2.89±0.11FG
6BA-60	50.33±0.88HIJ	25.67±1.20GH	15.29±0.49HIJK	2.60±0.10H
6BA-80	48.67±0.67IJK	24.67±0.88H	14.64±0.11IJKL	2.34±0.18I
6BA-100	42.67±1.45M	21.00±1.00I	12.73±0.52M	1.78±0.48J
NAA-10	59.00±1.00CD	38.33±1.20BC	18.22±0.46CDE	3.28±0.13DE
NAA-20	57.33±0.67DEF	32.00±1.15DE	17.09±0.30EFG	2.91±0.09F
NAA-40	53.67±0.67FGH	29.00±1.15EFG	15.66±0.28GHIJ	2.66±0.08GH
NAA-60	51.33±0.88GHIJ	28.33±0.67EFGH	15.27±0.24HIJK	2.44±0.05HI
NAA-80	47.67±0.33JKL	27.33±0.33FGH	14.37±0.15JKL	2.30±0.20I
BR-5	52.33±1.20GHI	31.33±0.67DE	16.19±0.38FGH	2.91±0.10F
BR-10	51.33±0.88GHIJ	30.00±0.58EFG	15.70±0.34GHIJ	2.67±0.09GH
BR-15	48.67±0.67IJK	29.67±1.33EFG	15.29±0.36HIJK	2.45±0.15HI
BR-20	46.33±0.67KLM	27.00±1.00FGH	14.18±0.33KL	1.98±0.05J
BR-25	44.33±1.20LM	25.00±1.00H	13.67±0.43LM	1.91±0.06J

Note: different lowercase and uppercase letters indicate values that are significantly different at $p < 0.05$ and $p < 0.01$, respectively. Values are the means of three biological replicates ± standard error. Different capital letters in each row indicate significant differences as determined by the analysis of variance, $p < 0.01$

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FLOOD HAZARDS IN THE CHI RIVER BASIN, THAILAND: IMPACT MANAGEMENT OF CLIMATE CHANGE

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Abstract. The Chi River Basin (CRB) is one of the major river basins in Thailand. Flooding often occurs in this floodplain, which has a profound effect on human life and property. Currently, climate change has a high impact on river discharges and therefore on floods. Hence, the management of flooding to minimize impacts due to climate change is essential and a priority at national and regional levels. The purpose of this study is to provide a quantitative understanding of the impacts of climate change on hydrological regimes and to provide a guideline for coping with the consequences. In this paper, General Circulation Model (GCM) outputs, the Statistical DownScaling Model (SDSM), the Soil and Water Assessment Tool (SWAT) model, the Hydrological Engineering Centre - River Analysis System (HEC-RAS) program, and the Geographic Information System (GIS) were used to determine areas vulnerable to flooding due to climate change. A flood hazard map was developed for the Lampao River Basin (LRB), which is a sub catchment of the CRB. According to the results, an appropriate flood management plan for the area was proposed. The plan involved structural and non-structural measures that integrated three methods of dealing with flood hazards which are modifying the hazards, moderating the impacts, and reducing the risks. Integrated flood management is a complex endeavor therefore computer-based tools that enable analysis of the whole system should be developed to implement the plan efficiently.
Keywords: *climate changes impact, downscaling, flood hazard, Chi River Basin, flood management*

Introduction

Climate change is one of the most critical issues in the world and in Thailand. Climate change has caused serious consequences in Thailand, such as higher surface temperatures, floods, droughts, severe storms and sea level rise (Sudtida, 2012). Over the past 30 years, there were more than 50 droughts and floods in Thailand (DEQP, 2016), and flooding is always a major problem in Thailand. Therefore, understanding the impact of climate change on flooding is vital to overcome this problem.

According to the Intergovernmental Panel on Climate Change (IPCC), General Circulation Model (GCM) can “reproduce features of the past climates and climate changes” (Randall et al., 2007). GCM are the best tools to estimate future global climate changes resulting from the continuous increase in the concentration of greenhouse gases in the atmosphere (Busuioc et al., 2001; Dibike and Coulibaly, 2005). The output of GCM can assist in understanding climate and in forecasting climate change. To simulate sub-grid scale phenomena, hydrological models are necessary, and such hydrological models require input data at a similar scale. These data are generally provided by converting the GCM outputs into a reliable regional hydrological time series at the selected catchment scale. Usually, “downscaling” techniques are used to convert GCM outputs into the local meteorological variables required for reliable hydrological modelling (Dibike and Coulibaly, 2005; Huntingford et al., 2006).

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In this study, daily precipitation series for specific area have to be obtained, therefore, the Statistical DownScaling Model (SDSM), which is a decision support tool for assessing local climate change impacts using a robust statistical downscaling technique, was selected (Kafatos, 2012). The statistically downscaled GCM outputs and the atmospheric circulation indices, as well as humidity variables derived from the CGCM3 model, were used to downscale daily precipitation series for the upper catchment of the Chi River Basin (CRB). The generated climate scenarios were then applied to drive the distributed Soil and Water Assessment Tool (SWAT) model. Changes in the modelled daily flow regime between current and future climate scenarios were compared and analysed. The impact of climate change on precipitation in the upper catchment of the Lampao River Basin (LRB), which is one of the CRB's sub-catchments, was investigated as a case study representing the Chi River Basin.

The analysed results were adopted to manage the impact of flood hazards, which concerns communities, the economy, the environment and human health. An appropriate flood management plan of the area was proposed. A flood mitigation plan was developed for critical disaster situations that affect life and property. Proper measures were presented to reduce the impact of flood hazards. The plan involves multiple projects that fall under one of two categories, i.e., hard engineering and soft engineering. The proposed plan is divided into the following:

- Early stage management (before the rainy season)
- During the occurrence of disasters (during the rainy season)
- Reconstruction plan (after disasters occur)

Materials and Methods

Study Area and Data Used

The Lampao sub-basin of the Chi River Basin for investigation in this study due to its frequency of flooding. The Chi River Basin has an area of 49.477 km^2 and is divided into 20 watersheds or catchment basins, which represent 29.10% of the northeast area of Thailand. The geographical location is between latitude 15˚ 30′ and - 17˚ 30′ north, and longitude 101˚ 30′ and - 104˚ 30′ east (Kuntiyawichai, 2011), as shown in *(Fig. 1).*

The topography of the Chi River Basin (CRB) includes tall mountains in the east and north from the Phu Phan mountain range. The west includes the Dong Phaya Yen mountain, which is the origin of the Chi River and several other major rivers. The central area is flat to undulating, and there is a small hill in the south of the basin. The major tributaries in the Chi River Basin include Nam Prom, Nam Chen, Nam Pong, and Lampao. Reservoirs in the basin include Ubol Ratana Dam, Chulabhorn Dam, Lum Nam Pung Dam, and Lampao Dam. The average precipitation is 1,028 mm/year. Approximately 6.7 million people live in this area.

The economies of the Chi River Basin depend on farming and animal husbandry. The income per person is 80.000-100.000 Baht/year (2.280-2.860 US dollars/year) (NSO, 2016).

According to recommendations of the Intergovernmental Panel on Climate Change (IPCC), the baseline used in this study is the standard normal for the period 1961-1990, as described by the standard of the World Meteorological Organization (IPCC-TGCIA, 1999). Data from two GCM simulation models, i.e., CGCM3 and HadCM3, were used

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in this investigation. The predictor variables for CGCM3 and HadCM3, described on a grid box, are provided in *Table 1*.

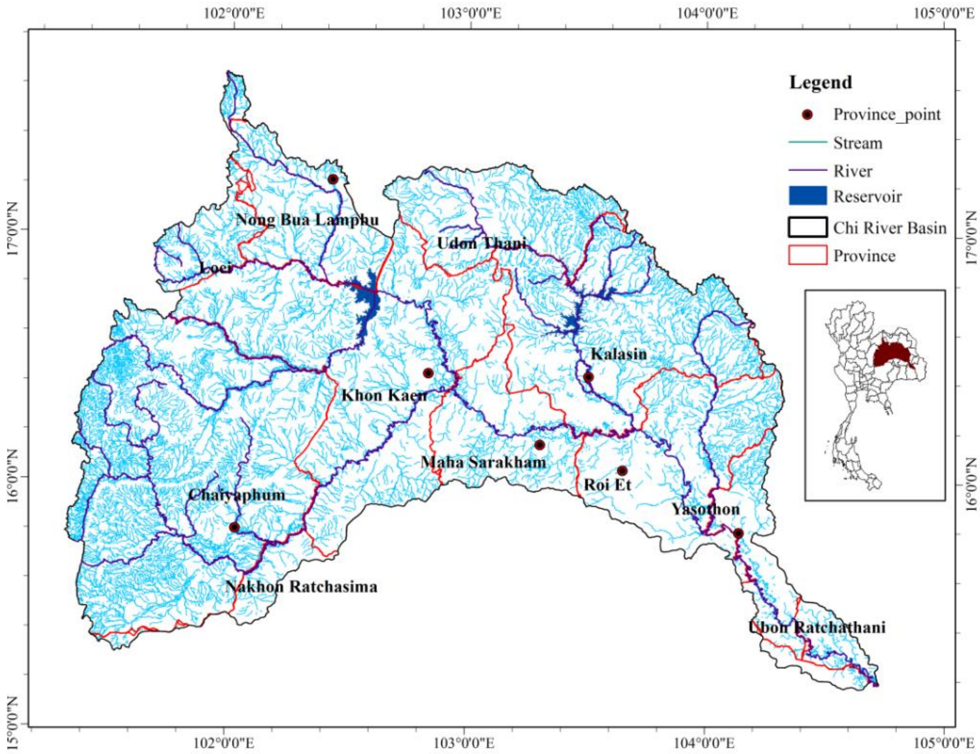

Figure 1. *Chi River Basin*

Table 1. *Grid positions of the Chi River Basin*

No.	Station Name	Code	Grid Position CGCM3	Grid Position HadCM3
1	Chaiyaphum	403201	28X_20Y	28X_29Y
2	Khonkaen	381201	28X_20Y	28X_28Y
3	Roiet	405201	29X_20Y	29X_29Y

Three weather stations of the Thailand Meteorological Department (TMD), as detailed in *Table 2*, located in the study area (*Fig. 2* and *Fig.* 3), provided daily precipitation data for CGCM3 and HadCM3. The past 50 years of data that cover the period suggested by the IPCC are available from the three stations.

Table 2. *Weather station locations*

No.	Station Name	Basin	Latitude	Longitude	Level (MSL.)
1	Chaiyaphum	Chi	15˚48′00″	102˚02′00″	180
2	Khonkaen	Chi	16˚27′48″	102˚47′12″	165
3	Roiet	Chi	16˚03′00″	103˚41′00″	140

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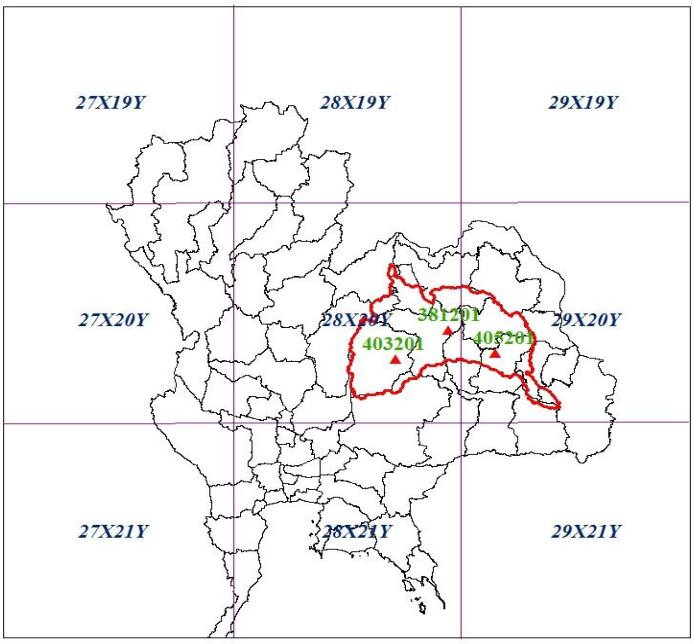

Figure 2. *Location of the weather station in CGCM3*

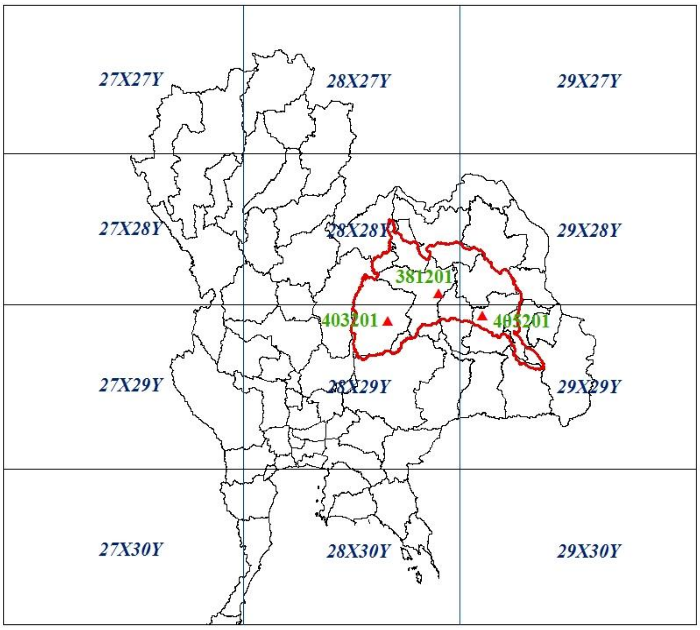

Figure 3. *Location of the weather station in HadCM3*

Hydrological data for the hydrological models include the following:

- Daily rainfall data from 17 TMD stations in the Lampao River Basin and nearby areas.
- Daily runoff, water level and cross section of the Lampao River from 8 stream gauge stations of the Royal Irrigation Department (RID) in the area.

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- Flood area from the Geo-Informatics and Space Technology Development Agency (Public Organization) – GISTDA.

Thus, the damage value from flooding can be evaluated in the flood area through the simulation results of the hydrological models. Damage includes loss of life and property, damage to buildings and other structures and flood health effects. These data were collected from the Department of Disaster Prevention and Mitigation.

Based on the flood risk and damage value, a flood management plan was developed to reduce the impact. This plan was compared to the existing plan of the Royal Irrigation Department that is adopted presently.

Method

The methodology of the study is shown in *Fig. 4* and is described in detail as follows:

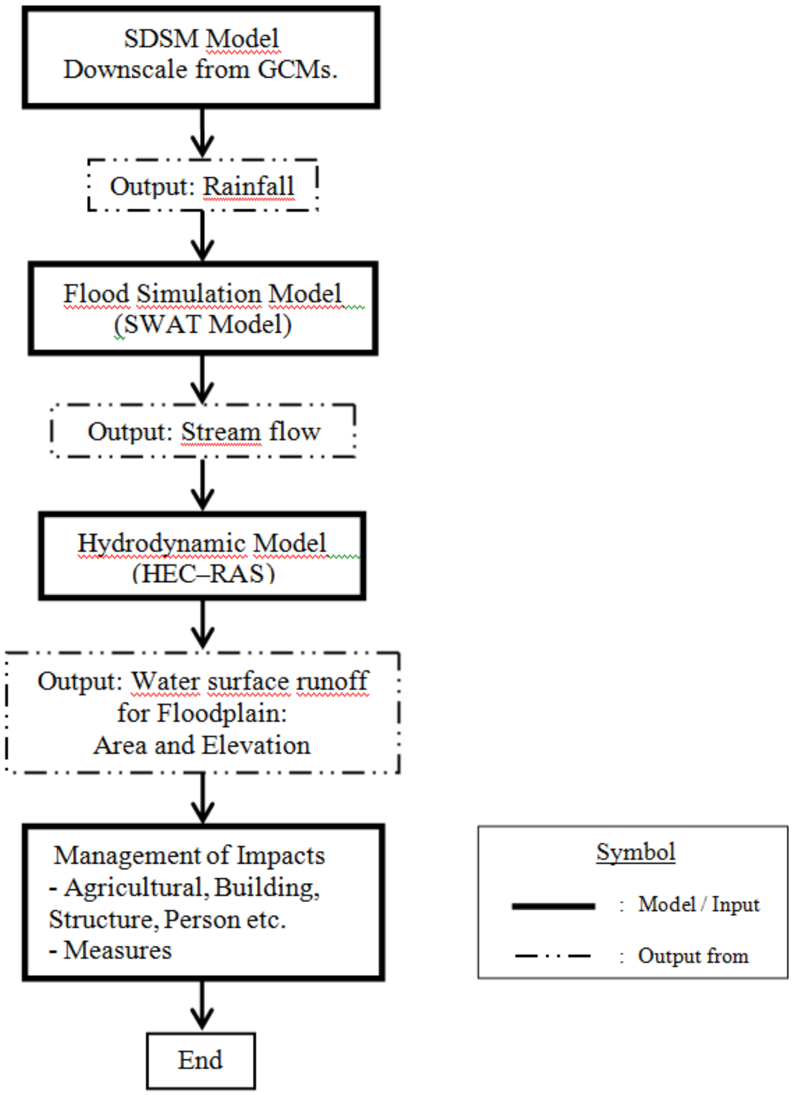

Figure 4. *Study flow chart*

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Statistical DownScaling Model (SDSM)

The Statistical DownScaling Model (SDSM) is a hybrid of the multiple linear regression and stochastic downscaling model developed by Rob Wilby and Christian Dawson (Harpham and Wilby, 2005; Wilby and Dawson, 2007). It is a freely available decision support tool for assessing local climate change impacts using a robust statistical downscaling technique. In the SDSM downscaling, a multiple linear regression model is developed between selected large-scale predictor variables and local-scale predictands such as temperature and precipitation. The SDSM uses a conditional process to downscale precipitation. Local precipitation amounts depend on wet/dry day occurrences.

GCMs (CGCM3 and HadCM3) approved by the Intergovernmental Panel on Climate Change (IPCC) were selected to construct climate scenarios. Data from these two international GCMs (CGCM3 and HadCM3) were obtained from the web site of the Canadian Climate Impacts and Scenarios project (http://www.cics.uvic.ca/scenarios). The source of scaling uncertainties is treated by downscaling the GCM outputs with the Statistical DownScaling Model (SDSM) (Wilby et al., 2001). This model is applied using conditional means involving stochastic weather generators.

The method used is a well-recognized statistical downscaling tool that has been made available to the broader climate change impact study community via the Canadian Climate Impact Scenarios (CCIS) project (Dibike and Coulibaly, 2005). The past 30 years of data represent the current climate (1961-1990), as recommended by the IPCC; the first 15 years are used for calibrating the regression model; and the remaining years of the data are used to validate the model.

This study applied the SDSM to predict future climate change in the Chi River Basin over 20 years, 50 years and 80 years. Changes in rainfall from the effects of climate change are obtained and used as the input in the hydrological model.

Hydrological Model

The Soil and Water Assessment Tool (SWAT) model, which has gained international acceptance as a robust interdisciplinary catchment modelling tool to quantify the impact of land management practices in large, complex catchments, was used to estimate the stream flow in LRB, because SWAT is a physically based model that is able to predict the impact of land use changes on water in a catchment and land use on a basin scale over long periods of time (Sun and Cornish, 2005). For the hydrodynamic model, the Hydrological Engineering Centre - River Analysis System (HEC-RAS) which is an accepted model to estimate surface runoff, was used (HEC, 2002).

The stream flow data measured at a stream gauging station located at Nong Mung (E.75) on the Lampao River was employed to calibrate the SWAT model. The observed data were split for calibration (2006-2009) and validation (2010-2013) purposes. Several statistical measures were used to evaluate the simulation accuracy, such as the Nash-Sutcliffe coefficient, the Root Mean Square Error, Goodness of fit and the Average Observance. Thereafter, the best calibrated model parameters were assigned to simulate stream flow with different rainfall scenarios from the SDSM to predict the tributary inflows with the probability of occurrence of flood inundation at all selected points on the Lampao River.

These stream flows were used as the input of HEC River Analysis System (HEC-RAS) model to determine the overflow depth of water to land subject to flooding.

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Flood Hazard

The flood depth was considered as the hazard indicator in this study. Therefore, the flood hazard categorization was adopted based on the induced flood depth. The flood hazard management model, as shown in *Fig. 5*, was proposed to reduce the impact of flood hazards.

Flood depth hazard maps were constructed based on the specific maximum flood depth with the probability of non-exceedance at 20%, 50%, and 80%. These maps are very useful for flood control and flood hazard management.

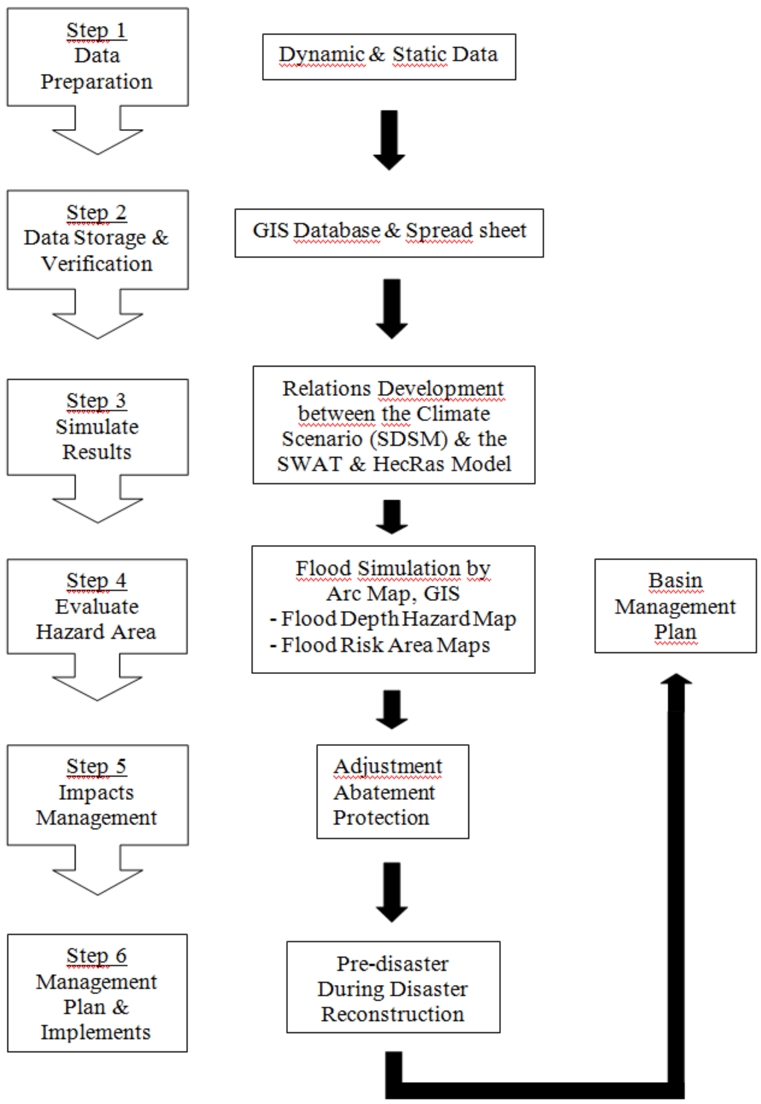

Figure 5. *Flood management model*

Results and Discussion

Choice of Predictor Variables

The National Centre for Environmental Prediction (NCEP) provides re-analysis data and GCM data used by SDSM. The data are available from 1961to -1990.

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Comparison of the predictor variables of the two GCMs, i.e., CGCM3 and HadCM3, can be selected by the entry screening method to obtain the least error results and the least used predictor variables. Large-scale predictor variables representing the current climate conditions, derived from the NCEP reanalysis data sets, were used to investigate the percentage of variance in each predictand-predictor pair. The most relevant predictor variables from the downscaling experiments from three weather stations in the area, i.e., Chaiyaphum (CPM), Khonkaen (KKN) and Roiet (RET), were determined and are shown in *Table 3*.

Table 3. *The relevant NCEP predictors from downscaling*

Predictors No.	NCEP (CGCM3)			NCEP (HadCM3)		
	Stations			Stations		
	CPM	KKN	RET	CPM	KKN	RET
ncepshumgl.dat	✓	✓	✓			
ncepp_vgl.dat	✓					
nceps500gl.dat	✓		✓			
ncepp8zhgl.dat			✓			
nceps850gl.dat			✓			
ncepr500as.dat				✓	✓	
ncepp5_zas.dat				✓		✓
ncepshumas.dat				✓		✓
ncepp_vas.dat						✓

SDSM Model Calibration and Validation

The coefficient of the multiple linear regression equation parameters that relate the large-scale atmospheric variables derived from NCEP and the local-scale variables was obtained by model calibration. The temporal resolution of the downscaling model for precipitation downscaling was specified as daily for Chaiyaphum, Khonkaen and Roiet.

From the 30 years of data, representing the current climate conditions, the first 15 years of data (1961-1975) were considered during calibration of the regression model while the remaining 15 years (1976-1990) were used to validate the model. *Table 4* shows the statistical results of CGCM3 and HadCM3 from three stations. From the NCEP selected variables, the results show that the CPM station (403201) prefers CGCM3 to HadCM3. For the KKN station (381201), HadCM3 provides better results than CGCM3. In addition, for the RET station (405201), CGCM3 is better than HadCM3. Therefore, CGCM3 was chosen for rainfall forecasting in the Chi River Basin (CRB).

Rainfall Forecast

The prediction of future rainfall was conducted for three periods, i.e., 2011-2040, 2041-2070 and 2071-2100. The prediction results were compared with rainfall observations in the years 1961-2010. The statistical evaluation results for the validation of SDSM are also shown in *Table 4*. The results are quite accurate and acceptable.

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Table 5 and *Fig. 6* show the computed results in the prediction of rainfall using CGCM3 and the baseline observation.

Table 4. *Statistical evaluation results for the validation of SDSM*

Statistic		Station 403201 (CPM) CG	403201 (CPM) Had	381201 (KKN) CG	381201 (KKN) Had	405201 (RET) CG	405201 (RET) Had
Mean	R^2	0.991	0.992	0.991	0.992	0.960	0.943
	Avg. Obs.	3.234	3.234	3.234	3.234	3.945	3.945
	RMSE	0.805	0.137	0.805	0.137	0.570	0.055
	Nash. Coef.	0.912	0.997	0.912	0.997	0.973	1.000
Maximum	R^2	0.714	0.528	0.714	0.528	0.701	0.661
	Avg. Obs.	84.550	78.717	84.550	78.717	89.850	89.850
	RMSE	16.253	13.942	16.253	13.942	29.637	20.480
	Nash. Coef.	0.767	0.824	0.767	0.824	0.752	0.882
Variance	R^2	0.881	0.901	0.881	0.901	0.891	0.890
	Avg. Obs.	94.110	98.277	94.110	98.277	134.316	134.316
	RMSE	33.230	18.977	33.230	18.977	61.809	32.044
	Nash. Coef.	0.830	0.941	0.830	0.941	0.786	0.943
Percentile	R^2	0.984	0.970	0.984	0.970	0.969	0.953
	Avg. Obs.	17.180	17.013	17.180	17.013	20.297	20.297
	RMSE	4.206	1.921	4.206	1.921	5.157	2.492
	Nash. Coef.	0.918	0.983	0.918	0.983	0.915	0.980
Sum	R^2	0.977	0.995	0.977	0.995	0.960	0.942
	Avg. Obs.	117.894	99.311	117.894	99.311	120.420	120.420
	RMSE	16.384	2.376	16.384	2.376	17.820	1.854
	Nash. Coef.	0.974	0.999	0.974	0.999	0.999	0.999

Table 5. *Precipitation forecast from the CGCM3 model*

Station Code	Model Grid CGCM3	Average Annual Rainfall (mm.) 1961-2010 Baseline	2011-2040	2041-2070	2071-2100
381201 (KKN)	28X20Y	1,227.72	1,421.25	1,453.22	1,497.70
405201 (RET)	29X20Y	1,375.30	2,316.81	2,757.65	3,579.56

In *Table 5*, the forecast results indicate that from now to the year 2100, rainfall in the Chi River Basin will increase due to climate change. Compared with the baseline, the rainfall at the KKN station (381201) during 2011-2040, 2041-2070 and 2071-2100 will increase by 15.76%, 18.37% and 21.99%, respectively. In addition, at the RET station (405201), a 68.46%, 100.51% and 160.27% increase in rainfall will occur. This indicates that the potential of flooding should increase in this area the future.

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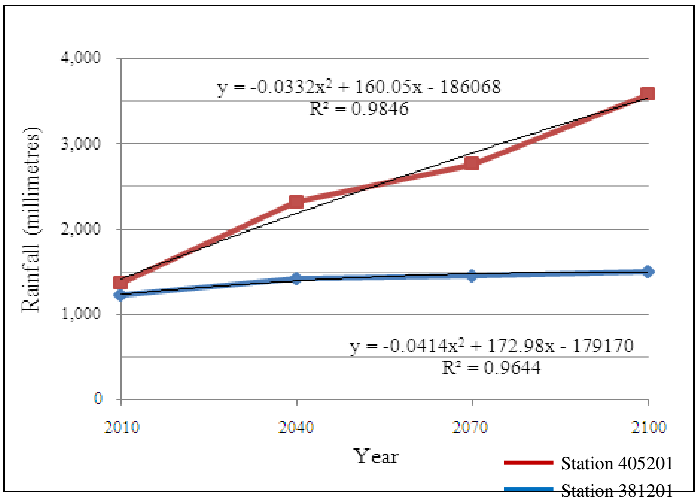

***Figure 6.** Precipitation forecast in Chi River Basin*

The average annual rainfall between 2010 and 2100 can be predicted from the equations given in *Fig. 6*. The equation representing the KKN station (381201) is $y = -0.0414x^2 + 172.98x - 179{,}170$ with $R^2 = 0.9644$, where y is the average annual rainfall (millimetres) and x is the year. In addition, the average annual rainfall at the RET station (405201) can be obtained by the equation y = -0.0332x2 + 160.05x -186,068 with $R^2 = 0.9846$.

SWAT Model Calibration and Validation

The historical discharge records from the E.75 stream gauge station on the Lampao River at Kalasin province from 2006 to 2013 were split into two sets of data. The first is 2006-2009 for calibration, and the second is 2010-2013 for validation of the SWAT model. Model calibration was conducted by comparing the SWAT simulated results with the observed discharge monthly. The simulated results of the monthly stream flow and the observed stream flow are compared for calibration in *Fig. 7* and for validation in *Fig. 8*.

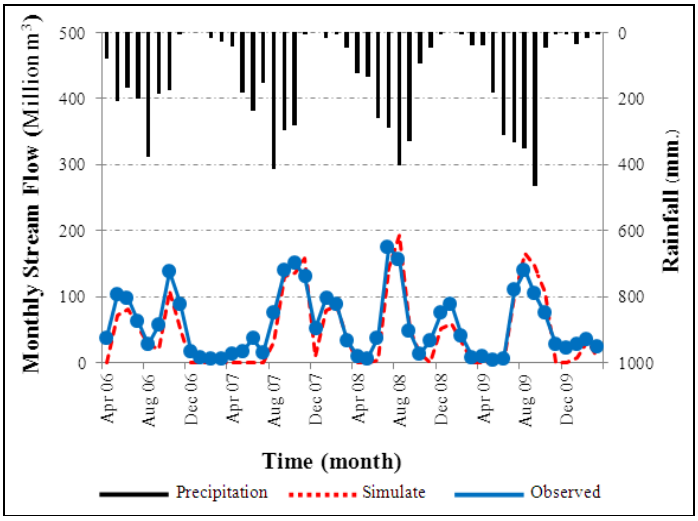

***Figure 7.** Calibration results of the SWAT model (2006-2009)*

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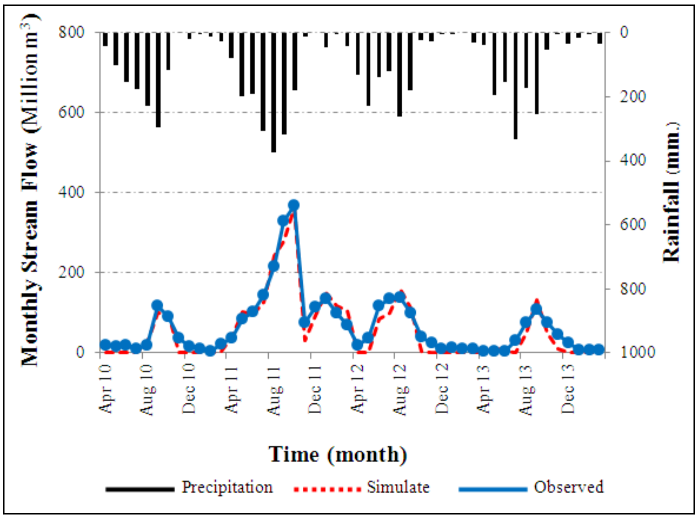

Figure 8. *Validation results of the SWAT model (2010-2013)*

From these figures, the simulated and observed monthly stream flow agree quite well; therefore, this SWAT model can be used to determine hydrological processes in this river basin. The evaluation of the statistical measurement error in the calibration and validation of the SWAT model is given in *Table 6*.

Table 6. *Statistical evaluation of the measurement error in the calibration and validation of the SWAT model*

Evaluation Statistics	Station E.75	
	Calibration	Validation
R^2	0.873	0.940
Avg.Obs.	57.681	67.514
RMSE	23.247	22.633
Nash. Coef.	0.769	0.916

To obtain the stream flow, the downscale predicted precipitation results were input to the SWAT model to simulate the stream flow in different scenarios. The simulation results are shown in *Table 7* and *Fig. 9*. The maximum daily stream flows during 2011-2040, 2041-2070, and 2071-2100 are 220.79, 239.44, and 258.09 m^3/s, respectively. Compared with the baseline, the maximum daily stream flow will decrease by 30.18% in the next ninety years. From the simulation model, the average annual stream flow, average annual water volume, and the highest annual stream flow show an increasing trend, but the maximum daily stream flow from the model is lower than the maximum daily stream flow recorded due to a large flood in this area in 2011.

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***Table 7.** Simulation results of the SWAT model for the Lampao River at station E.75*

Period		Average Annual Stream Flow	Increase	Average Annual Water Volume	Increase	Max. Daily Stream Flow
		(m^3/s)	(%)	(megacentimetres)	(%)	(m^3/s)
Baseline	2006-2011	28.39	-	898.36	-	369.67
2020	2011-2040	82.47	190.50%	2,606.92	190.19%	220.79
2050	2041-2070	89.50	215.27%	2,829.33	214.94%	239.44
2080	2071-2100	96.58	240.21%	3,053.02	239.84%	258.09

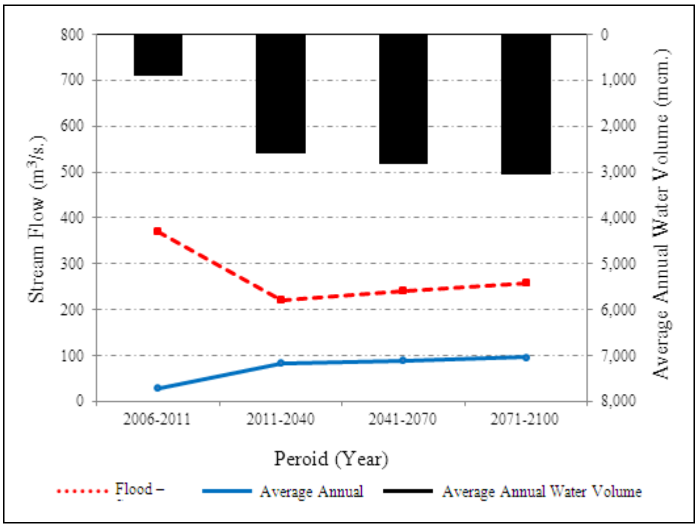

***Figure 9.** Summary of simulation results of SWAT model*

Flood Area

Data of the maximum daily stream flow and flooding in the area were extracted from the Geo-Informatics and Space Technology Development Agency (Public Organization) – GISTDA (GISTDA, 2012). The accumulated annual stream flow and flood area from 2006 to 2011 are given in *Table 8*.

***Table 8.** Flood area of the Lampao River Basin*

Year	Flood Area (km^2)	Max Daily Stream Flow (m^3/s)
2006	41.29	71.50
2007	154.67	350.19
2008	34.71	89.88
2009	15.20	133.36
2010	102.94	157.40
2011	192.88	369.67
Average	90.28	193.06

To determine the flood area for the next ninety years, the stream flow simulation results were input to HEC-RAS to compute the resulting water surface elevation. These

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elevations were mapped in ArcGIS to form a flood inundation map. As a result of the flood area shown in *Table 9*, the maximum flood area due to climate change in the next ninety years is less than the maximum flood area in the past.

Table 9. *Flood area caused by climate change*

Period		Maximum daily Stream Flow (m^3/s)	Flood Area (km^2)
Baseline	2006-2011	369.67	192.88
2020	2011-2040	220.79	115.20
2050	2041-2070	239.44	124.93
2080	2071-2100	258.09	134.66

The results show an increase in the maximum daily stream flow from 2012 to 2100 as well as the flood area. When compared to the baseline flood area, the future maximum flood area is lower due to one extraordinary flood in 2011. If the average daily stream flow and flood area are considered, the future runoff and flood area is larger than the baseline, and an increasing trend for the future is noticed.

The flood depth is considered to be the most important indicator of the intensity of the flood hazard. Therefore, the inundation area is classified into three classes according flood depth, as shown in *Table 10*. The flood height represents the severity, in which a low level causes inconvenience, a medium level causes the inundation of low lying areas and requires the evacuation of some areas, and a high level is widespread flooding causing extensive damage to people. A flood hazard map was constructed according to the inundation area, water height classification, and risk.

Table 10. *Flood stage classification*

Level	Water Height (cm.)
High	> 40
Medium	20-40
Low	< 20

The river overflow data consists of the maximum water height and flood duration collected in the field, as shown in *Table 11*. The runoff-water height relationship can be established from these data.

Table 11. *Duration of flood and maximum annual water height of river overflow*

Year	Duration of flood (days)	Maximum Annual Water Height (cm)
2006	-	10.00
2007	20	50.00
2008	-	15.00
2009	-	20.00
2010	-	25.00
2011	18	55.00
Average	6.33	29.17

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Flood Management

Currently, there are many agencies in Thailand involved in water management. This leads to inconsistent and non-integrated water management plans and actions. At least twelve government agencies are responsible for flood control, and there is a little cooperation among these agencies. Most of the existing flood plans are emergency plans developed independently by some of these government agencies. Thus, an integrated flood plan consisting of preparation, response, recovery, and mitigation of flood risk needs to be developed. Therefore, a flood management plan to reduce the impact on communities, the economy, the environment and health for this area was constructed as follows. It is important that lessons from the past, knowledge and technology, effective communication and applications are considered. The plan contains three phases:

- Early stage management (before the rainy season)
- During the occurrence of disasters (during the rainy season)
- Reconstruction plan (after disasters occur)

Three factors, i.e., human behaviour and decisions, probabilities of flood event and damage, and government responsibility are integrated in the plan. Traditionally, flood prevention has relied on civil engineering works. In recent years, soft measures, such as flood warning systems, flood-plain zoning, insurance, etc., have become more recognized. The management approach varies in the different phases. In this study, the optimization of the two means has been considered. The objective is to protect people and property from the damaging impacts of floods resulting climate change.

To cope with the problem, the relationship between the stream flow and flood area was determined. The major flood in 2011, which is incident in this area, which has the highest of daily stream flow 369.67 m^3/s and the highest of flood area 192.88 km^2, was set as the calibrated data. The result of the input data to the HEC-RAS model is shown in *Fig. 10*. *Table 12* shows the simulation results from the HEC-RAS model, from which the relationship between the flood area and stream flow in the Lampao River Basin can be constructed.

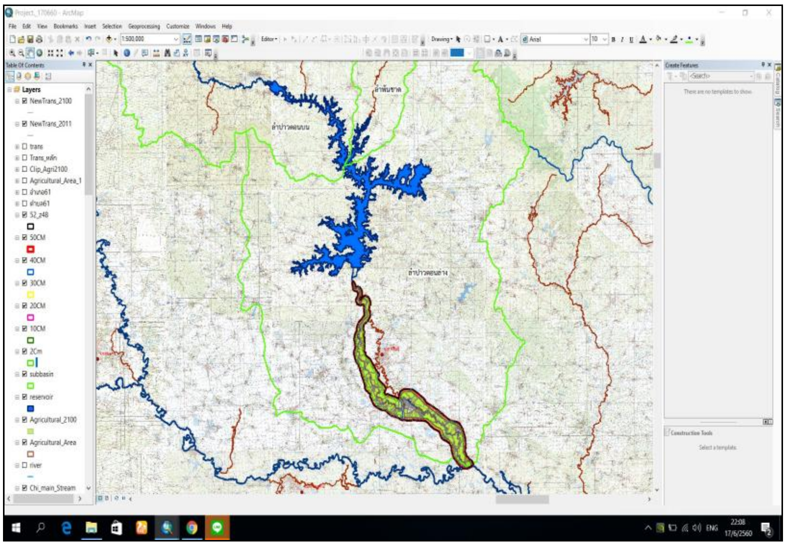

Figure 10. *The flood area from the HEC-RAS model superimposed on a topographic map 1:50,000*

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***Table 12.** Flood area and stream flow in the Lampao River Basin*

Flood Area (km^2)	Stream Flow (m^3/s)	Flood Area (km^2)	Stream Flow (m^3/s)
0.00	209.53	114.24	343.15
1.70	250.94	115.93	344.12
9.39	274.72	121.86	347.14
10.56	276.63	141.29	351.99
24.56	288.57	162.82	359.50
48.86	305.18	168.39	361.01
80.34	328.55	180.37	365.50
80.41	328.69	191.41	368.29
102.70	339.15	192.88	369.67

From *Fig. 11*, the relationship between the stream flow and flood area in the Lampao River Basin can be represented as

$$Y = 0.0118X^2 - 5.6557X + 673.84 \qquad \text{(Eq. 1)}$$

where X is the stream flow (m^3/s) and Y is the flood area (km^2), with $R^2 = 0.9959$.

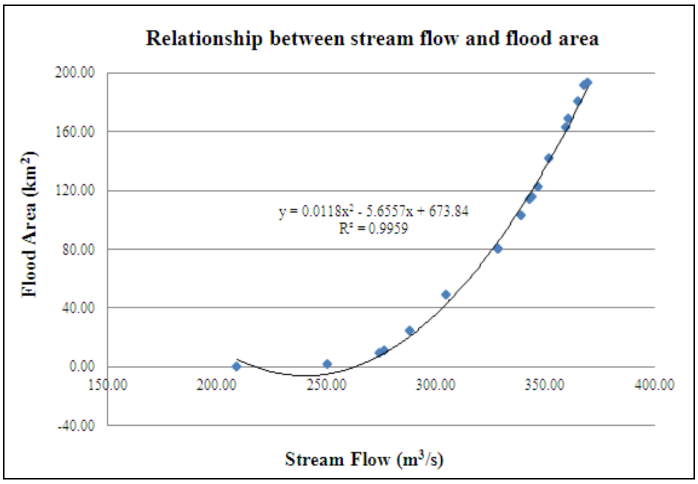

***Figure 11.** Relationship between the stream flow and flood area*

The statistical data of flooding in the past are in agreement with this equation. Due to field operation, the stream flow can be obtained from the water height, which is more practical to measure. Therefore, the relationship between the stream flow and flood level was constructed, as shown in *Fig. 12*, as follows:

$$Y = 0.0015X^2 - 0.4916X + 33.821 \qquad \text{(Eq. 2)}$$

where X is the stream flow (m^3/s) and Y is the flood level (cm), with $R^2 = 0.9752$.

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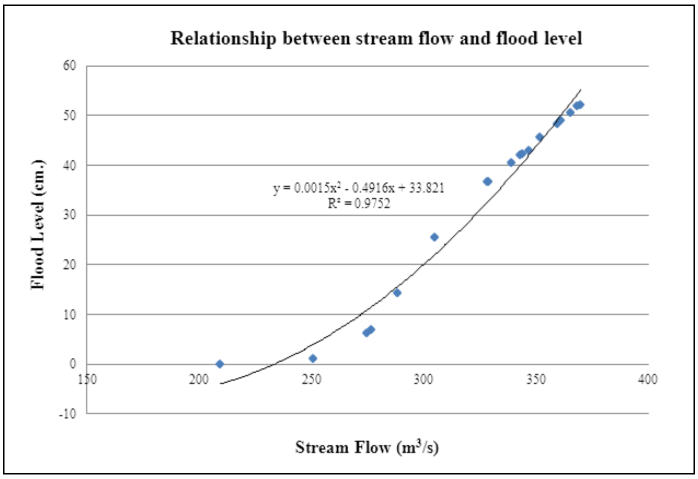

Figure 12. *Relationship between the stream flow and flood level*

The probability of flooding can be determined from the flood level, as shown in *Fig. 13*, and correlations of the flood probability to the flood level, stream flow and flood area are shown in *Table 13*. Through comparison with the flood stage classification in *Table 8*, a flood risk area map can be obtained.

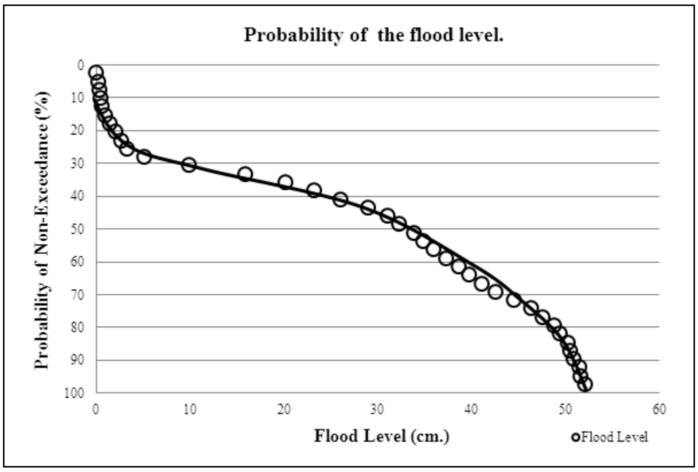

Figure 13. *Probability of the flood level*

Table 13. *Probability of non-exceedance of the flood level*

Probability of Non-exceedance (%)	Flood level (H) (cm)	Stream Flow (Q) (m^3/s)	Flood Area (A) (km^2)
0.00	0.00	209.53	0.00
10.00	3.23	261.04	4.96
30.00	9.68	281.13	15.84
50.00	27.78	310.05	55.42
70.00	43.33	347.75	124.29
90.00	50.36	364.93	178.85

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Impact Management of the Flood Hazard

Floods impact both individuals and communities, and have social, economic, and environmental consequences. In Thailand, the governmental mechanisms are available for managing flood hazards. Because of the inefficiency of integration and collaboration, flood hazards still are an unsolved problem. Therefore, it is urgent to develop a method to effectively manage flood hazards. This study proposes the flood management plan to cope with the problem. The plan is aimed at assisting the stakeholder to undertake their flood management responsibilities and ensure that suitable measures are be implemented. The plan comprises three phases, i.e., early stage management, during the disaster and reconstruction. Four general strategies, i.e., modify the loss, modify vulnerability, modify the event and modify the cause, were applied in this plan. Activities according to this plan are presented as follows:

Early Stage Management

It is essential that communities recognize flooding as part of their environment. They must be aware of the flood potential. In this stage, four activities should be applied.

- Mapping of potential flood zones

  Flood hazard mapping and risk assessment were conducted to identify priority areas and high-risk zones. The inundation areas, corresponding to the flood depth, should be determined.
- Asset management

  Flood mitigation assets are maintained in a fit-for-purpose state to ensure they work as designed during a flood. All flood defence structures, including waterways, should have ongoing inspection and maintenance programs. The asset should be managed in accordance with the asset management guidelines and asset management plans. Asset conditions should be assessed and reported annually.
- Planning controls

  Local agencies should to consider planning permits for land use and development projects with regard to area conditions to ensure that flood control systems continue to function properly. Providing necessary information to stakeholders on the importance of appropriate design and mitigation of flooding is a good practice. Any new developments should be adequately studied and designed to protect the community and environment from flooding. The assessment of planning permit applications should focus on flooding impacts and risks. The continued assessment of flood risks at the planning permit application stage has resulted in more appropriate development in known flood risk areas. Integration and communication among responsible agencies are essential.
- Community education and awareness

  Steps to create awareness of preparedness measures within the community should be taken. Public participation and public perception are important components of success. Flood preparedness education programs should be implemented. These programs will ensure that stakeholders are aware of flood impacts and appropriate flood response actions. Appropriate behaviour during the occurrence of flood events is an important element in the minimization of losses. Notification of the amendment, along with flood maps and up-to-date data, makes flooding information readily accessible to the community.

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During the Occurrence of Disasters

Two activities should be implemented as follows:

- Flood warning system

Flood warning systems and services aim to reduce losses and impacts caused by flooding and are important flood mitigation measures. Two important outcomes from flood warning systems are informing those at risk of flooding and appropriate actions being taken by those at risk. Currently there is no warning system in the area of this study; alerts of the water height according to flood hazard maps by radio, telephone, short message service (SMS) and internet, including social media, are proposed. The water height should be reported every hour. The warning message should be sent when the stream flow reaches 200 m^3/s, and reports should continue at 15-minute intervals. Instruction should also be included in all messages.

- Flood emergency planning

Flood emergency planning is crucial to ensure an effective, proactive emergency response to flooding. All stakeholders must be recognized and well informed of this plan, which should be applied within the legislated framework. The plan should facilitate a consistent and coordinated approach to flood response within the risk area in the lead up to, during and immediately after a flood event. All arrangements should detail in the plan. This plan should be put into action by the community and government agencies. Communication and consultation are essential for success.

Reconstruction

This stage involves decisions regarding the return to normal activities after a period of flooding. After a flood occurs, impacts should be evaluated, and mitigation plans should be implemented. Post-disaster recovery measures should be taken as follows:

- Rebuilding

Immediately after the flood, houses, public services, infrastructures, e.g., roads, electricity, and the water supply, are rebuilt or repaired.

- Insurance and tax adjustments

Assistance and relief in the form of low-cost and subsidized insurance and taxes should be provided, along with low-cost loans. This would relieve the short-term stresses of the community.

- Income generating activities

Supporting income generating activities of members of the community is a high priority. The community should return to a normal activity and income by any means as fast as possible.

- Flood plan evaluation

To reduce the risk of floods in the future, lessons from past floods should be considered. Collecting important data, such as stream flow, flood areas, flood duration, emergency plan implementation, and mental health surveys is essential. A revise flood plan should be generated for the next early stage management step.

In this area, the impacts and risks to people and property were studied. To mitigate climate change in the future, applying the proposed flood management plan will produce less impact as seen in *Table 14*.

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Table 14. Risk to people and property in the Lampao River Basin during a flood event

Impacted	Unit	Current	Future	Decrease(%)
Number of People	People	7,079	6,358	10.19
Number of Properties	Family	1,863	1,287	30.92
Agriculture	Square Kilometre	163.69	112.96	30.99
Infrastructure	Square Kilometre	1.80	1.23	31.67

Catastrophe Stress of Flooding in the Lampao River Basin

A survey of catastrophe stress of flooding was conducted in the Lampao River Basin sometime after when a major flood in 2011. The standard inquires of catastrophe stress, as determined by Department of Mental Health, Ministry of Public Health (ST5), were conducted. Effects on mental health from the stress of flooding were assessed in a community survey of 900 people, and the results are given in *Table 15*.

Table 15. Catastrophe stress of flooding in the Lampao River Basin

Stress level	Number of People (people)	Percentage (%)
Minor	127	14.11
Gentle	325	36.11
Considerable	313	34.78
Severe	135	15.00
Sum	900	100.00

Flood victims face several problems simultaneously, such as inconvenienced living, loss of property, loss of income, etc. These issues can lead to stress, anxiety, and depression. Mental illness treatment should be provided for an indefinite time until the community mental health recovers. Community mental health is a good indicator for evaluating the success of a flood management plan and should be considered in the revised plan.

Conclusion

Climate change poses significant risk to the Chi River Basin. This study responds to the needs of decision makers to plan for flooding caused by climate change. A quantitative study of the impacts of climate change based on hydrological regimes and of managing the impacts is presented. A sub-catchment, i.e., the Lampao River Basin, was used to yield detailed results for the entire basin. An increase in precipitation was observed, with the consequence of increasing the risk of rain-induced flooding. Regional climate scenarios, particularly for precipitation, were derived from SDSM to downscale climatic data for stream flow modeling of the Lampao River Basin. The results show that the SDSM provides adequate downscaled precipitation data using CGCM3 predictors. Stream flows are obtained from imputing different scenarios of precipitation data to the SWAT and HEC-RAS models and using the results to develop

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flood hazard maps. A flood management plan was presented as a useful tool so that all sectors can be aware of vulnerabilities and more efficiently cope with flooding.

While the plan was developed with simulated flood-related legislation and supporting policies and strategies, it recognizes that no single approach comprises an effective response to flood management issues. It also recognizes that it is not possible and to eliminate areas subject to flooding within the region, as a residual risk will remain.

Development of computer-based tools that enable analysis of the whole system, evaluation of the consequences of strategic intervention and coordination of intervention activities as a flood decision support system could be a beneficial research.

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CHANGES IN THE FLORISTIC COMPOSITION AND ECOLOGY OF RUDERAL FLORA OF THE TOWN OF KOSOVSKA MITROVICA, SERBIA FOR A PERIOD OF 20 YEARS

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Abstract. The paper is concerned with the results of the ruderal flora investigation carried out in the vicinity of the town of Kosovska Mitrovica (Serbia) and its surroundings, in different urban and suburban habitats, and is based on the copious floristic researches conducted between 1995 and 1996 and repeated in 2016. The total number of 444 taxa was reported in the course of 2016. Not only was reported the presence of 386 taxa in the same areas between 1995 and 1996, but also 58 new taxa were recorded in recent field explorations. The ruderal flora composition in Kosovska Mitrovica area has changed by 13.06% in the past 20 years. Detailed taxonomic, ecological, and phyto-geographical analyses were provided for the discovered synanthropic flora. Special attention was paid to the appearance of new invasive species unregistered 20 years ago, but which, due to the more intensive anthropogenic influence, have become more diverse in number and frequency in the investigated areas.
Keywords: *urban habitats, ruderal flora, ecological analysis, invasive plants, Kosovska Mitrovica*

Introduction

Urbanization has created new ecosystems that harbor specialized flora adapted to anthropogenic alterations (Neto et al., 2015). The ecological approach considers a city/town as an ecosystem. "Ruderal" biocenoses exist in all cities. The term "ruderata" (from Lat. rudus: rubble, ruins) refers to a specific habitat. The definition connects ruderal plants with their habitats: they grow in places (such as rubble) which are strongly disturbed by man, but not cultivated (Sukopp, 2002). The habitats with modified physical and chemical soil properties have lost their resemblance to primary land type and are located in the vicinity of settlements and around all types of urban buildings and infrastructures (Prodanović et al., 2008). Other terms used for the flora in human settlements are synanthropic, synurban and nitrophile flora (Pavlović-Muratspahić et al., 2010). Anthropogenic factors are of essential importance in the formation, survival, distribution, diversity and dynamics of this type of flora/vegetation (Jarić et al., 2011).

The flora of urban habitats has long been recognized as being rich in species (Stešević and Jovanović, 2008). Urbanization transforms floras through a series of filters that change: (i) habitat availability;(ii) the spatial arrangement of habitats; (iii) the pool of plant species; and (iv) evolutionary selection pressures on populations

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persisting in the urban environment. Habitat transformation and fragmentation are anthropogenic filters present in most ecosystems, while the strong influences of human preference and urban environmental conditions are unique to cities (Williams et al., 2008). The development and importance of this spontaneously developed plant cover are often underestimated in urban ecosystem by constant human efforts to destroy and curb it by giving way to cultivated plants. Ruderal plant species are often considered as invasive weeds; some have medicinal value while others have unknown utility. Regardless of its specific use, the ruderal flora grows rapidly and thus serves as study material for many botanical subareas and provides an opportunity to study the development of vegetation when most plants are annuals. The ecology of ruderal plants also reinforces the importance of these plants because they can vegetate, bloom, and fructify with high efficiency, allowing them to be used for the recovery of degraded areas (Neto et al., 2015).

Ruderal and synanthropic (synurban) floras, very young and dynamic floristic assemblages, as well as the corresponding vegetation units, have recently drawn attention of scientists throughout the world (Jovanović, 1997). The research of synanthropic flora of various urban areas (cities, towns and smaller settlements) has had a long tradition. The interest in urban floras can be attributed to the fact that cities are remarkably ample with species due to a high habitat diversity and abundance of alien species. Outside Europe, urbanization and its consequences have been intensively studied in North America (Celesti-Grapow et al., 2006). The first investigations in the field of urban ecology in Europe focused on single habitat types (old settlements, ruins, gardens, and parks), while comprehensive studies of urban ecosystems were started in the 1970′s (Sukopp, 2002). In Serbia, as a part of territory of former Yugoslavia, similarly to other European countries, such studies were conducted in the second half of the 19th century (Šajinović, 1968; Slavnić, 1951, 1960, 1961; Marković, 1964, 1978, 1984; Matvejeva, 1982; Jarić, 2000), while special interest in the urban/ruderal flora and vegetation appeared in works of Jovanović (1997), Jovanović and Mitrović (1998), Stankovic-Kalezić (2007), Stanković-Kalezić et al. (2008; 2009).

The floristic research of ruderal weed was conducted to determine accurate data about the qualitative composition of ruderal flora and the quantitative presence of certain species on ecologically diverse surfaces of urban and suburban areas of the town of Kosovska Mitrovica (Serbia). The purpose of the paper was to identify possible changes in the floristic composition of ruderal flora for a period of 20 years, which might provide data on the succession planting and fully introspect ecology of each individual plant species in the investigated area.

Materials and Methods

The investigation was carried out in the central area of the town of Kosovska Mitrovica (Serbia) and its surroundings, on various urban and suburban ruderal surfaces within more than 15 kilometers. During the growing season of 1995-1996, field researches were conducted on 28 randomly chosen sampling sites (trodden and nitrified ruderal places, nitrified ruderal non-trodden habitats, hygrophilic ruderal habitats, covered disposal areas). During 2016 the researches were continued in the majority of the previously investigated areas but it was virtually impossible to provide a detailed map of already studied probing areas because they had absolutely changed due to extreme anthropogenic influences (i.e. urbanization). Likewise, as the town had

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extended to new areas, another two probing surfaces were added during floristic investigations in 2016. The majority of collected plant material is kept in the Herbarium at the Institute of Botany and the Botanical Garden of Faculty of Biology, University of Belgrade (BEOU).

The collected plants were determined on the basis of modern floristic literature: The Flora of Serbia: Josifović, ed. 1970-1977; Sarić and Diklić, eds. 1989 and The Flora of Europe: Tutin, ed. 1964-1980. The nomenclature was adjusted to Euro+Med Plantbase (2006) and The Plant List (2013). The life forms of plants were determined by applying Ellenberg and Muller-Dambois method (1967), with Stevanović's supplements (1992) and elaborations based on the conditions in Serbia. Stevanović's (1992a) phyto-geographical classification was the starting point for determination and phyto analysis of floral elements. The prevalence was calculated by the number of sites where the target species occurred divided by the total number (30) of sampling sites.

Study area

The town of Kosovska Mitrovica, with its surroundings, lies between 42° 53' north latitude, 20° 52'east longitude, and 496-510 altitude, and stretches to the farthest northern point of Kosovo Valley (*Fig. 1).* It is situated in the alluvial area and on the terraces of the Ibar and Sitnica Rivers, with the volcanic cup of Zvečan dominating the city. The largest part of the territory is considered mountaineous. More than half of the city and its surroundings consists of brown shallow soil with poor fertility. It should be emphasized that, influenced by heavy industry, many types of soil have undergone degradation and "enrichment" with various waste products that reduced their fertility. The climate is marked as moderate-continental. Mitrovica is located among Kopaonik and Rogozna mountains, hence the annual precipitation is low (600 mm on average).

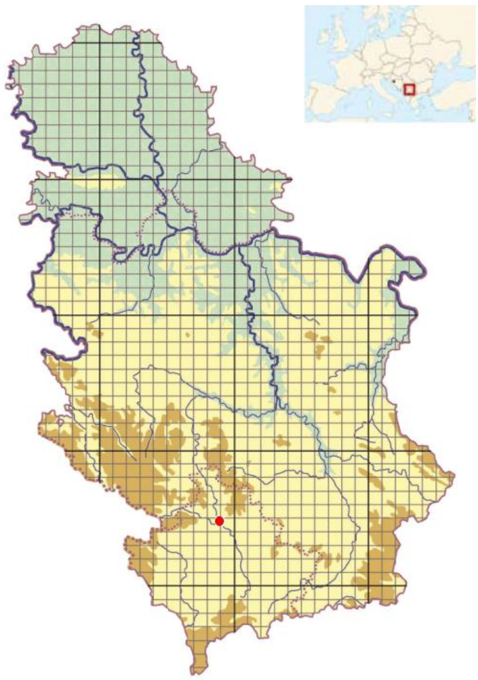

Figure 1. *Geographical position of investigated area (town of Kosovska Mitrovica) in Serbia (marked with red point)*

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Results and Discussion

Taxonomic spectrum of the ruderal flora in the town of Kosovska Mitrovica

Ruderal species are plants commony grown in urban areas and are best adapted to man-altered environments. Floristic researches in different types of ruderal habitats in the city proper of Kosovska Mitrovica and its surroundings, within the range of 15 kilometers, discovered 444 species of vascular plants representing 271 genera and 58 families (*Table 1*).

The study presents a continuation of the research, whose results were published in 2008 (Prodanović et al., 2008). The presence of 386 taxa was reported in the same areas between 1995 and 1996, and 58 new taxa were recorded in recent field explorations. It is important to emphasize that not all collected species are ruderal, even though they can be traced in various anthropogenic habitats. A certain number of species are found in habitats of primary or secondary forms of vegetation, such as forest, meadows or completely segetal (field) species. These species apperently present either the debris of the original primary communities that were prior to ruderal communities or they appeared afterwards.

Newly recorded taxa include: *Allium flavum* L., *Chaerophyllum bulbosum* L., *Vinca minor* L., *Hedera helix* L., *Asparagus tenuifolius* Lam., *Ambrosia artemisifolia* L., *Cota austriaca* (Jacq.) Sch. Bip, *Symphyotrichum novi-belgii* (L.) G.L.Nesom, *Symphyotrichum salignum* (Willd.) G.L.Nesom, *Calendula officinalis* L., *Echinops sphaerocephalus* L., *Filago germanica* (L.) Huds., *Helianthus tuberosus* L., *Lactuca muralis (L.) Gaertn*., *Lactuca perennis* L., *Solidago gigantea* Aiton, *Tanacetum corymbosum* (L.) Sch.Bip., *Catalpa bignonioides* Walter., *Anchusa azurea* Mill., *Cynoglossum creticum* Mill., *Nonnea pulla* DC., *Arabidopsis thaliana* (L.) Heynh., *Camelina sativa* (L.) Crantz., *Sisymbrium officinale* (L.) Scop., *Sisymbrium orientale* L., *Blitum bonus-henricus* (L.) Rchb., *Cuscuta europaea* L., *Echinocystis lobata* (Michx.) Torr.& A. Gray, *Carex panicea* L., *Shoenoplectus lacuster* (L.) Palla, *Euphorbia taurensis* All., *Amorpha fruticosa* L., *Trifolium arvense* L., *Vicia tenuifolia* Roth., *Vicia tetrasperma* (L.) Schreb., *Geranium columbinum* L., *Leonurus cardiaca* L., *Malva neglecta* Wallr., *Maclura pomifera* (Raf.) C.K. Schneid., *Fraxinus ornus* L., *Phytolacca americana* L., *Platanus orientalis* L., *Arrhenatherum elatius* (L.) J. Presl & C. Presl, *Bromus arvensis* L., *Poa trivialis* L., *Reseda phyteuma* L., *Sherardia arvensis* L., *Populus alba* L., *Populus nigra* L., *Salix alba* L., *Comandra umbellata* (L.) Nutt., *Acer negundo* L., *Acer pseudoplatanus* L., *Acer saccharinum* L., *Antirrhinum majus* L., *Veronica praecox* All., *Veronica serpyllifolia* L. and *Viola arvensis* Murray.

The taxonomic spectrum of the vascular flora in the city area of Kosovska Mitrovica includes 3 classes, 58 families, 271 genera and 444 species and subspecies. *Dicotyledones*, with 51 families, 231 genera (49,09%) and 384 taxa (86,03%), are richer in number than *Monocotyledones* which include 6 families (1,35 %), 41 genera (9,23%) and 60 taxa (13,51%), whereas horsetail *(Equisetinae)* is present only with two species of *Equisetaceae.* According to the researches of Pyšek et al. (2009) the most common families in the urban areas of Europe are: *Asteraceae, Poaceae, Rosaceae, Fabaceae* and *Brassicaceae,* which, along with ruderal species, comprise farming weed and invasive species; their number is positively related to the urbanization level (Pyšek, 1998).

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Table 1. *Overview of ruderal flora of Kosovska Mitrovica (Serbia) with its life forms, floristic elements and prevalence (%x100)*

Family/Species	**Life forms**	**Floristic element**	**Prevalence**
ALLICEAE			
Allium flavum L.	v-a Mes-Mac G bulb	apen(s)-ilir-balk-dac-pan	3,3%
Allium vineale L.	v-a Mes-Meg G bulb	med-subm-pan-atl-ce	6,6%
ADOXACEAE			
Sambucus ebulus L.	a Alt G rad scap/a H scap	se-med-subm-pont-j.sib-or-tur	10%
Sambucus nigra L.	fo dec Mi P scap	se-med-subm-pont-j.sib	10%
AMARANTHACEAE			
Amaranthus albus L.	a Meg T scap	adv (sam-sram)	6,6%
Amaranthus blitoides S. Watson	a Mes-Meg T rept	adv (sam)	10%
Amaranthus retroflexus L.	a Mes-Alt T scap	adv (sam)	30%
APIACEAE			
Aegopodium podagraria L.	a Meg-Alt G rhiz scap	se-med-subm-pont-j.c.sib	13,3%
Anthriscus caucalis M. Bieb.	a Mes-Meg T scap	subevr-az.	23,3%
Anthriscus cerefolium (L.) Hoffm. var. trichospermus Endl.	a Meg T scap	i.subm-pan-pont-tur	26,6%
Anthriscus sylvestris (L.) Hoffm.	a Meg-Alt H scap	se-med-subm-pont-j.sib-i.afr	23,3%
Bifora radians M.Bieb.	v-a Mes-Meg T scap	c.ev-c.i.med-subm-pont-or-tur	16,6%
Chaerophyllum bulbosum L.	a Mes-Alt H/T scap bienn	c.ev-med-subm-pont	16,6%
Conium maculatum L.	a Meg H scap bienn	se-med-subm-pont-j.sr-sib-or-tur-ca-i.j.afr	23,3%
Daucus carota L.	a Meg H scap/a T scap	se-med-pont-or-tur-i.afr	36,6%
Eryngium campestre L.	a Mes-Meg H scap	med-subm-pont	43,3%
Falcaria vulgaris Bernh.	a Mes-Meg T scap	se(z.ev-j.sarm)-pont-or-tur-ca	23,3%
Foeniculum vulgare Mill.	a Meg-Alt H scap	adv (med-or-tur)	10%
Heracleum sphondylium L.	a Meg-Alt H scap	se-ev (bor)-med-subm-pont-j.c.sib	36,6%
Orlaya grandiflora (L.) Hoffm.	a Meg T scap	c.ev-med-subm-pan-z.pont	30%
Pastinaca sativa L.	a Meg H scap bienn	se-med-pont-j.sib	30%
Scandix pecten-veneris L.	v Mes-Meg T scap	se-med-subm-or-tur-ca	43,3%
Torilis arvensis (Huds.) Link	a Meg T scap	kosm (ev-med)	16,6%
APOCYNACEAE			
Vinca minor L.	Mi-Mes Ch suff rept	adv (se, kult)	6,6%
ARALIACEAE			
Hedera helix L.	semp S lig	atl-se-med-subm (ev)	13,3%

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ARISTOLOCHIACEAE			
Aristolochia clematitis L.	a Mes-Meg G rad scap	subm-pont	23,3%
ASPARAGACEAE			
Asparagus tenuifolius Lam.	a Meg G rhiz caesp	c.subm-pan-z.pont	3,3%
ASTERACEAE			
Achillea millefolium L.	a Meg H scap	evr (bor-submerid)	60%
Ambrosia artemisifolia L.	Aut Meg T scap	adv(sam)	6,6%
Anthemis arvensis L.	a Mes-Meg T scap/a H scapp	med-subm	26,6%
Cota austriaca (Jacq.) Sch. Bip	a Mes-Meg T scap bienn	pont-pan	36,6%
Cota tinctoria (L.) J. Gay	a Meg H scap bienn	se-med-subm-or-pont	19%
Arctium lappa L.	aut Meg-Alt H scap bienn	evr (temp-submerid)	43,3%
Artemisia absinthium L.	Meg Ch suff caesp	evr (subbor-merid)	30%
Artemisia scoparia Waldst.& Kit.	aut Meg-Alt H scap bienn	evr (subbor-submerid)	36,6%
Artemisia vulgaris L.	aut Meg- Alt H scap	evr-sam (subbor-merid)	60%
Symphyotrichum novi-belgii (L.) G.L.Nesom	aut Meg-Alt H scap	adv (sam)	10%
Symphyotrichum salignum (Willd.) G.L.Nesom	aut Meg-Alt H scap	adv (sam)	10%
Bellis perennis L.	a Mes H ros	se-med-subm	60%
Bidens tripartitus L.	aut Mes-Alt T scap	evr (subbor-temp)	6,6%
Calendula officinalis L.	a Mes-Meg T scap	adv (med, kult)	10%
Carduus acanthoides L.	a Meg-Alt H scap bienn	se-med-subm-pont-j.c.sib-or-tur	23,3%
Carlina vulgaris L.	a Meg H scap	se-subm-pont-j.sib	3,3%
Centaurea jacea L.	a Meg-Alt H scap	evr (subbor-submerid)	40%
Centaurea orientalis L.	a Meg-Alt H scap	pan-z.pont	36,6%
Centaurea scabiosa L.	a Meg-Alt H scap	se-med-pont-j.c.sib-tur	36,6%
Centaurea solstitialis L.	a Meg T scap	med-subm-or-pont-j.sib-tur	50%
Centaurea stoebe L. subsp. australis (Pančić ex A.Kern.) Greuter	a Meg H scap	pan-z.pont	53,3%
Chondrilla juncea L.	a Meg-Alt H scap	med-subm-or-pont-j.sib-tur	10%
Cichorium intybus L.	a-aut Meg-Alt H scap	kosm (evr)	50%
Cirsium arvense (L.) Scop.	a Meg-Alt G rad scap	evr (subbor-merid)	46,6%
Cirsium candelabrum Griseb.	a Alt H scap bienn	balk. (end)	23,3%
Cirsium creticum (Lam.) d'Urv.	a Meg H scap bienn	med-subm	20%
Cirsium vulgare (Savi) Ten.	a Meg-Alt H scap bienn	evr (subbor-merid)	16,6%
Crepis biennis L.	a Meg-Alt H scap bienn	se-subm-pont	10%
Crepis nicaeensis Pers.	a Mes-Meg T scap/a H scap bienn	med-subm-or	10%

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Crepis rhoeadifolia M.B. Fiori et Paol.	a Meg-Alt H scap bienn	se-subm-pont	30%
Crepis sancta (L.) Bornm.	v-a Mes-Meg T ros/H ros	pont	20%
Crepis setosa Haller	a Mes-Meg T scap	c.i.med-subm-or-z.pont	20%
Cyanus segetum Hill.	a Mes-Meg T scap	kosm (med)	36,6%
Echinops sphaerocephalus L.	a Meg-Alt H scap	se-subm-pont-j.sib	23,3%
Erigeron acer L.	a Mes-Meg T scap	evr-az	50%
Erigeron canadensis L.	a Meg-Alt T scap	adv (sam)	53,3%
Eupatorium cannabinum L.	a Meg-Alt H scap	se-med-subm-pont-j.sib	16,6%
Filago germanica (L.) Huds.	a Mes T scap	se-med-subm-or-pont-j.sib-tur	10%
Galinsoga parviflora Cav.	a Mes-Meg T scap	adv (jam)	40%
Helianthus tuberosus L.	a Meg-Alt G bulb	adv (sram)	10%
Inula britannica L.	a Mes-Meg H scap	evr (temp-merid)	16,6%
Inula conyzae (Griess.) DC.	a Meg-Alt H scap bienn/a H scap	ev-z.az	16,6%
Lactuca muralis (L.) Gaertn.	a Mes-Meg H scap	evr (bor-submerid)	30%
Lactuca perennis L.	a Mes-Me H scap	subm	30%
Lactuca serriola L.	a Meg-Alt H scap bienn/a T scap	evr (subbor-merid)-i.afr (boreosubtrop)	66,6%
Lactuca viminea (L.) J Presl.& C.Presl.	a Meg-Alt H scap bienn	med-subm-pont-pan-ir (w)-boh-burgd.	16,6%
Lapsana communis L.	a Meg-Alt T scap	se-med-subm-pont-or-j.sib-ca	10%
Leontodon crispus DC, ex Nyman	a Mi-Mes H ros	med-i.subm-pont-j.sib	13,3%
Leucanthemum vulgare (Vaill.) Lam.	v-aut Mes-Meg H scap	evr (bor-merid)	30%
Matricaria chamomilla L.	a Mi-Mes T scap	kosm (subm)	56,6%
Onopordum acanthium L.	a Meg-Alt H scap bienn	evr (temp-merid)	16,6%
Petasites hybridus (L.) G.Gaertn., B.Mey &Schaerb	a Mes-Meg G rad	evr (bor-submerid)	16,6%
Picris hieracioides L.	a Meg-Alt H scap bienn/a H scap	evr (temp-merid)	23,3%
Pilosella bauhinii (Schult) Arv.-Touv.	a Mes H ros rept	c.ev.-sarm-pont-j.sr.sib	40%
Pilosella officinarum Vaill.	a Mi-Mac H ros	SJEP	40%
Podospermum laciniatum (L.) DC.	a Mi-Meg T scap bienn/H scap	pont-c.az.-subm	23,3%
Pulicaria dysenterica (L.) Bernh.	a Mes Meg H scap	se-med-subm-pont-or-tur	16,6%
Senecio leucanthemifolium subsp. vernalis (Waldst.& Kit.) Greuter	v Mes-Meg T scap	ev-med-subm-or-pont-j.sib	30%
Senecio vulgaris L.	v-aut Mi-Meg T scap	kosm (evr)	43,3%
Solidago gigantea Aiton	a Meg-Alt H scap	adv (sam)	10%
Solidago virgaurea L.	a Meg-Alt H scap	evr-sam (bor-temp)	10%
Sonchus arvensis L. var. arvensis	a Meg-Alt H scap	kosm (evr)	23,3%

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Sonchus arvensis L. subsp.. uliginosus (M.Bieb.) Nyman	a Meg-Alt H scap	kosm (evr)	8-26,6%
Sonchus asper (L.) Hill. subsp. asper	a Meg-Alt T scap/a H scap bienn	kosm (med-subm)	9-30%
Sonchus asper (L.) Hill. subsp. glaucescens (Jord.) Ball	a Meg-Alt H scap bienn	ev.-z.az	9-30%
Sonchus oleraceus L.	a Meg-Alt T scap/a H scap bienn	kosm (med-subm)	33,3%
Tanacetum corymbosum (L.) Sch.Bip.	a Meg-Alt H scap	se-med-subm-pont-j.sib	10%
Tanacetum vulgare L.	a Meg-Alt H scap	evr (temp-merid)	23,3%
Taraxacum officinale F.H.Wigg.	v-aut Mes H ros	kosm (evr)	76,6%
Tragopogon dubius Scop.	a Mes-Meg H scap bienn	se-subm-pont-or	50%
Tragopogon dubius Scop. subsp. major (Jacq.) Vollm.	a Mes-Meg H scap bienn		50%
Tragopogon pratensis L.	a Meg H scap	subm (ev) pont-j.c.sib-tur	63,3%
Tripleurospermum inodorum (L.) Sch.Bip	a-aut Meg T scap/ H scap bienn	kosm (med-subm)	50%
Tussilago farfara L.	v Mi-Mes G rhiz	se-med-subm-pont-j.sib-ca	23,3%
Xanthium orientale subsp. italicum (Moretti) Greuter	a Meg-Alt T scap	adv (sam)	26,6%
Xanthium spinosum L.	a Mes-Meg T scap	kosm (jam)	33,3%
Xeranthemum annuum L.	a Mes-Meg T scap	med-subm-or-z.pont	43,3%
BIGNONIACEAE			
Catalpa bignonioides Walter.	v-a Mes P scap	subm	3,3%
BORAGINACEAE			
Aegonychon purpurocaeruleum (L.) Holub	v Mes-Meg H scap	pont-subm	20%
Anchusa azurea Mill.	a Mes-Meg H scap	med-subm-pan-pont-or	16,6%
Anchusa officinalis L.	a Meg H scap bienn/a H scap	se-i.subm-pan-z.pont	46,6%
Asperugo procumbens L.	v Mes-meg T scap	evr (bor-submerid)-sam (sin.)	10%
Buglossoides arvensis (L.) I.M.Johnst.	v-a N-Meg T scap	evr (subbor-merid)	16,6%
Cerinthe minor L.	v-a Mes-Meg H scap bienn/T scap	(ev)-med-subm-z.pont	23,3%
Cynoglossum creticum Mill.	a Mes-Meg H scap bienn	atl-med-subm-tur	16,6%
Cynoglossum officinale L.	a Mes-Meg H scap bienn	se-med-subm-pont-sr.sib	26,6%
Echium italicum L.	a-aut Meg H scao bienn	med-subm	20%
Echium vulgare L.	a Mes-Alt H scap bienn/a H scap	se-med-subm-pont-j.sib	30%
Heliotropium europaeum L.	a Mes-Meg T scap	med-subm-pan-z.pont	10%
Myosotis arvensis (L.) Hill.	a Mes H scap bienn/a T scap	se-med-subm-pan-pont-or	16,6%
Myosotis sparsiflora Pohl	v-a Mes-Meg T scap	se-z.subm-pont-j.sib	23,3%

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Nonnea pulla DC.	v-a Mes-Mac H/T scap bienn	subpont	16,6%
BRASSICACEAE			
Alliaria petiolata (M.Bieb.) Cavara & Grande	v-a Meg H scap bienn	ev-med-z.tur	30%
Alyssum alyssoides (L.) L.	v Mi-Mes T scap	med-subm-pont	16,6%
Alyssum margrafi O.E. Schulz ex Markgr.	a Mes-Meg H scap	balk (end)	3,3%
Alyssum turkestanicum Regel & Schmalh.	v Mi-Mes T scap	pan-pont-j.sib-or-tur	3,3%
Arabidopsis thaliana (L.) Heynh.	a Mi-Meg H ros bienn/a T ros-scap	ev-med-z.ca	13,3%
Armoracia rusticana P. Gaertn., B. Mey. & Scherb.	a meg-Alt G rad scap	adv (pont-kult)	3,3%
Barbarea vulgaris R. Br.	a Meg H scap	evr (subbor-merid)	16,6%
Berteroa incana (L.) DC.	a Mes H scap	se-pont-j.sib-tur	43,3%
Calepina irregularis (Asso) Thell.	a Mes-meg T scap	med-pont-tur	36,6%
Camelina sativa (L.) Crantz.	a Mes-Alt T scap	evr (temp-merid)	33,3%
Capsella bursa-pastoris (L.) Medik.	v-aut Mi-Meg T ros/H ros bienn	kosm (subm)	66,6%
Cardamine hirsuta L.	v-aut Mi-Mes T scap	kosm (evr)	33,3%
Cardaria draba (L.) Desv.	v-a Meg H scap	med-subm-pont-tur	63,3%
Conringia orientalis (L.) Dumort.	v-a Mes-meg T scap	i.med-i.subm-z.pont	30%
Coronopus squamatus (Forssk.) Asch.	v-a Mi-Mes T rept	kosm (med)	20%
Descurainia sophia (L.) Prantl	a Meg T scap/a H scap bienn	evr (temp-merid)	46,6%
Diplotaxis muralis (L.) DC.	v-a Mes T semiros/H semiros	se-subm	46,6%
Erophila verna (L.) Chevall.	v N-Mi T ros	med-pont-j.sib-tur	30%
Erysimum cuspidatum (M.Bieb..) DC.	a Meg T scap/a H scap bienn	i.subm-pan-z.pont-tur	36,6%
Erysimum diffusum Ehrh.	a Mes-Meg H scap bienn	pont-j.sib-tur	50%
Lepidium campestre (L.) W.T. Aiton	a Meg T scap/a H scap bienn	ev-subm-pont	6,6%
Lepidium ruderale L.	a Mes T scap	evr (temp-merid)	6,6%
Microthlaspi perfoliatum (L.) F. K. Mey.	v Mi-Mes T scap-semiros	med-subm-pont-tur	23,3%
Myagrum perfoliatum L.	v Mes T scap	i.subm-pont-tur	26,6%
Odontarrhena bertolonii subsp. scutarina (Nyár) Španiel, Al-Shehbaz, D.A. German & Marhold	a Mes-Meg H scap	balk (end)	30%
Raphanus raphanistrum L.	v-a Meg T scap	evr (temp-merid)	16,6%
Rorippa austriaca (Crantz) Besser	a Meg H scap	i.med-z.pont	30%
Rorippa pyrenaica (All.) Rchb.	a Mes H semiros-scap	subm (ev)	23,3%
Rorippa sylvestris subsp. kerneri (Menyh.) Soó	v-a Mi-Mes H scap bienn	panon	33,3%
Rorippa silvestris (L.) Besser	a Mi-Mes H scap	med-subm-pan-pont	43,3%
Sinapis alba L.	v-a Meg T scap	i.med-subm	46,6%
Sinapis arvensis L.	v-a Mes-Meg T scap	kosm (subm)	36,6%

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Sisymbrium loeselii L.	a Meg-Alt T scap	i.subm-pan-pont-tur	26,6%
Sisymbrium officinale (L.) Scop.	a Meg T scap	med-subm-pont-j.c.sib	46,6%
Sisymbrium orientale L.	a Meg T scap/ a H scap bienn	med-pont	40%
Thlaspi arvense L.	a Mes T scap	evr (temp-merid)	60%
CANNABACEAE			
Humulus lupulus L.	a SH herb	evr-sam (subbor-temp)	23,3%
CAPRIFOLIACEAE			
Dipsacus laciniatus L.	a Meg-Alt H scap bienn	c.ev-sarm-i.subm-pont-j.sib-or	16,6%
Knautia arvensis (L.) Coult	a Mes-Meg H scap/a H scap bienn	ev (boreo)-se-med-subm-pont-j.sib	16,6%
Scabiosa argentea L.	v-a Meg H scap bienn/H scap	i.subm-pan-z.pont	23,3%
Valerianella carinata Loisel.	v Mi-Mes T scap	atl-se-med-subm	33,3%
Valerianella locusta (L.) Laterr.	a Mes T scap	kosm (med)	26,6%
Valerianella turgida (Steven) Betcke	v Mes T scap	i.med-subm	23,3%
CARYOPHYLLACEAE			
Agrostemma githago L.	a Meg T scap	evr (temp-merid)	16,6%
Arenaria serpyllifolia L.	v-a Mi-mes T scap	ev-md-pont-tur	43,3%
Cerastium brachypetalum Pers.	a Mi-Mes T scap	ev-med-subm-z.pont	20%
Cerastium fontanum subsp. vulgare (Hartm.) Greuter & Burdet	v-a Mi-Mes H scap	kosm (evr)	13,3%
Cerastium glomeratum Thuill.	a Mi-Mes T scap	kosm (med)	23,3%
Herniaria glabra L.	v-a Mi-Mes T rept/H rept bienn	evr (subbor-submerid)	26,6%
Holosteum umbellatum L. var. umbellatum	v Mi-mes T scap	evr (temp-submerid)	40%
Moenchia mantica (L.) Bartl.	v-a Mes T scap	(ev)-c.i.med-c.i.subm-pan	20%
Myosoton aquaticum (L.) Moench.	a Mes-Meg H rept	evr (bor-subtemp)	13,3%
Petrorhagia saxifraga (L.) Link. f. cinerascens Th. Wolf.	a Mes H caesp	c.i.med-pan-z.pont	23,3%
Saponaria officinalis L.	a Meg H scap	se-med-pont-j.sib	16,6%
Scleranthus annuus L. subsp. polycarpos (Torn.) Thell.	v-aut Mi-Mes T scap/H bienn	evr (temp-submerid)	10%
Scleranthus perennis subsp. dichotomus (Schur) Nyman	v-aut Mi-Mes H scap	i.subm-pan	13,3%
Silene armeria L.	a Meg T scap/H scap bienn	evr-sam (temp-submerid)	20%
Silene conica L. subsp. conica Gusul.	v-a Mi-Mes T scap	evr (temp-submerid)	23,3%
Silene flos-cuculi (L.) Clairv.	a Meg H scap	se-ev (bor)-subm-pont-j.sib	13,3%

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Silene latifolia Poir.	a Meg H scap bienn/a H scap	evr (temp-submerid)	30%
Silene vulgaris (Moench) Garcke	a Meg H scap/a G rad	evr (bor-merid)	50%
Stellaria graminea L.	a Mes-Meg H scap	evr (bor-submerid)	20%
Stellaria media (L.) Cirillo	v-aut Mi T rept	kosm (med)	60%
CHENOPODIACEAE			
Atriplex patula L.	aut Meg-Alt T scap	evr-sam (subbor-merid)	10%
Atriplex rosea L.	a-aut Mes-Meg T scap	med-subm-pan-pont.j.sib-or	6,6%
Atriplex tatarica L.	a Meg-Alt T scap	evr (temp-merid)	13,3%
Bassia scoparia (L.) A.J. Scot	a Meg-Alt T scap	adv (ca)	3,3%
Blitum bonus-henricus (L.) Rchb.	a Mac H scap	ev-sam (bor-merid)	6,6%
Chenopodiastrum hybridum (L.) S. Fuentes & al.	a Mes-Meg T scap	evr (temp-merid)	13,3%
Chenopodiastrum murale (L.) S. Fuentes & al.	a Meg-Alt T scap	kosm (med)	23,3%
Chenopodium album L. var. album	a Meg-Alt T scap	evr (bor-merid)	60%
Chenopodium opulifolium W.D.J. Koch & Ziz	a Meg T scap	kosm (med)	43,3%
Chenopodium strictum Roth.	a Meg T scap	ev-z.az	33,3%
Chenopodium suecicum Murr var. viride (L.) Wahlenb	a Mes-Alt T scap	evr (temp-submerid)	26,6%
Dysphania botrys (L.) Mosyakin & Clemants	a Mes-Meg T scap	evr (temp-merid)	43,3%
Lipandra polysperma (L.) S. Fuentes & al.	a Meg T scap	evr	10%
Salsola tragus L. subsp. tragus	a Mes-Alt T scap	pan-pont-j.sib-tur-ca	3,3%
CISTACEAE			
Fumana procumbens (Dunal.) Gren.& Godr.	a Mes Ch suff rept	z.i.c.med-subm-se-z.pont-or	13,3%
CONVOLVULACEAE			
Calystegia sepium (L.) R.Br.	a SH herb	kosm (evr-sam)	16,6%
Convolvulus arvensis L.	a SG herb rhiz	kosm (med)	93,3%
Cuscuta epithymum (L.) L.	ST par	atl-se-med-subm-pan-pont-j.sib	23,3%
Cuscuta europaea L.	ST par	ev-az	30%
CRASSULACEAE			
Sedum acre L.	N-Mi Ch herb caesp succ	atl-se-med	6,6%
CUCURBITACEAE			
Bryonia alba L.	a SG tub herb	sarm-i.subm-pont-j.sib	13,3%
Echinocystis lobata (Michx.) Torr.& A. Gray	a St herb	adv (sam)	10%
CYPERACEAE			
Carex hirta L.	a Mes-Meg G rhiz caesp	ev-med-pont	23,3%

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Carex panicea L.	v-a Meg H caesp/rhiz	cirkumpolarni	20%
Carex vulpina L. f. nemorosa (Rebent.) Koch.	a Meg H caesp	evr (temp-submerid)	30%
Cyperus fuscus L.	a Mes emer Hyd T scap	evr (subbor-merid)	16,6%
Eleocharis palustris (L.) R.Br.	a Mes-Meg emer Hyd G rhiz	kosm (evr)	16,6%
Shoenoplectus lacuster (L.) Palla	a-aut Alt emer Hyd G rhiz	kosm	13,3%
EQUISETACEAE			
Equisetum arvense L.	a Mes-Meg G rhiz scap	evr-sam (bor-temp)	20%
Equisetum palustre L.	a Meg G rhiz	evr-sam (bor-temp)	13,3%
EUPHORBIACEAE			
Euphorbia cyparissias L.	a Mes-Meg H scap	atl-se-c.subm-pan-sarm	50%
Euphorbia taurensis All.	a Mes T scap	subm	33,3%
Euphorbia helioscopia L.	a Mi-Meg T scap	kosm (evr)	9-30%
Euphorbia seguieriana Neck. subsp. niciciana (Borbas ex Novak) Reich.	a Mes-Meg G rad caesp	evr (temp-submerid)	40%
Euphorbia stricta L.	v-a Mes-Meg T scap/H bienn	ev-z.az.	26,6%
FABACEAE			
Amorpha fruticosa L.	fo dec Mi P caesp	adv (s.amer)	6,6%
Anthyllis vulneraria L.	a Mes-Meg H scap	ev-med-subm-z.pont	3,3%
Astragalus cicer L. f. microphyllus (L.) Acherson et Graebn.	a Mes-Meg H scap	j.atl-subm-pont-sarm	6,6%
Astragalus glycyphyllos L.	a Mes-Meg H scap-rept	ev.i-subm-pont-j.sib-tur	13,3%
Astragalus hamosus L. f. multiflorus	v-a Mes-Meg T scap	med-subm-tur	3,3%
Dorycnium pentaphyllum subsp. herbaceum (Vill.) Bonnier & Layens	Mes Ch suff caesp	med (ap-balk) c.subm-pan-pont	23,3%
Galega officinalis L.	a Meg H scap	c.i.subm-pan-pont-or	13,3%
Genista tinctoria L.	Meg fo dec Ch suff caesp	se-sarm-subm-pont-j.c.sib	26,6%
Lathyrus aphaca L.	a Mes T scap/ST herb	med-subm-pont-j.tur	33,3%
Lathyrus sphaericus Retz.	a Mes-Meg T scap	med-subm	23,3%
Lathyrus tuberosus L.	a Meg G tub rept	se-subm-pont-j.sib-or-i.afr	36,6%
Lotus corniculatus L.	a Mes H scap	ev-med-pont-j.sib-or-i.afr	66,6%
Medicago arabica (L.) Huds.	a Meg T scap	med-subm-or-tur	56,6%
Medicago falcata L.	a Mes-Meg H scap	ev-med-subm-pont-j.sib	40%
Medicago lupulina L.	a Mes T scap/a H scap	evr (temp-merid)-i.afr	53,3%

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Medicago minima (L.) L.	a Mi-Mes T scap	evr (submerid-merid)-i.afr.	30%
Medicago orbicularis (L.) Bartal.	a Mes T scap	med-subm-or-tur	43,3%
Medicago rigidula (L.) All.	a Mi-Mes T scap	med-subm-or	46,6%
Medicago sativa L.	a Mes-Meg H scap	med-subm-or	33,3%
Melilotus albus Medik.	a Meg T scap/a H scap bienn	kosm (evr)	43,3%
Melilotus officinalis (L.) Lam.	a Meg-Alt H scap bienn	ev-subm-pont-j.sib-or-tur	60%
Onobrychis viciifolia Scop.	a Meg H scap	adv (med;kult)	16,6%
Ononis spinosa L.	fo dec Mes-Meg Ch suff caesp	atl-c.ev	23,3%
Ononis spinosa subsp. hircina (Jacq.) Gams	fo dec Mes Ch suff caesp	i.subm-pan-sarm-pont-j.sib	20%
Robinia pseudoacacia L.	fo dec Mes P scap	adv (sam)	13,3%
Securigera varia (L.) Lassen	a Meg H scap	se-med-subm-pont-or	30%
Trifolium arvense L.	a Mes T scap/a H scap bienn	ev-med-pont-j.sib	40%
Trifolium campestre Schreb.	a Mes T scap	ev-med-subm-z.pont-or-tur	50%
Trifolium dubium Sibth.	v-a Mi-Mes T scap	ev-z.az	70%
Trifolium fragiferum L.	a Mes H rept	ev-med-subm-pont-j.sib-or-tur	60%
Trifolium incarnatum L.	a Mes-Meg T scap	subatl-med-subm	40%
Trifolium patens Schreb.	a Mes-Meg T scap	(ev) med-subm	63,3%
Trifolium pratense L.	a Mes H scap	ev-med-subm-pont-j.sib	33,3%
Trifolium repens L.	a Mi H rept	kosm (evr)	50%
Trifolium resupinatum L.	a Mes T scap	med-subm-or-tur	36,6%
Vicia cracca L. var. linearis Petern	a Meg-Alt H scap/SH herb	evr (bor-merid)	53,3%
Vicia grandiflora Scop.	a Meg T scap/ST herb	i.subm-pan-z.pont	13,3%
Vicia hirsuta (L.) Gray f. fissa (Frol) Beck.	a Mes-Meg T scap/ST herb	ev-med-pont-j.sib	43,3%
Vicia lutea L.	v-a Mes-Meg T scap	z.c.med-subm-or-tur	40%
Vicia pannonica Crantz	a Mes-Meg T scap/ST herb	ev-med-subm-z.pont	43,3%
Vicia pannonica subsp. striata (M. Bieb.) Nyman	a Mes-Meg T scap	i.med-i.subm-or	43,3%
Vicia sativa L.	a Mes-Meg T scap/ST herb	kosm (med;kult)	50%
Vicia sativa subsp. nigra (L.) Ehrh.	v-a Mes-Meg T scap/H scap bienn	ev-z.az	36,6%
Vicia tenuifolia Roth.	a Mac-Alt H scap/SH herb	ev-med-z.az	30%
Vicia tetrasperma (L.) Schreb.	Mes ST herb	kosm (med)	26,6%
Vicia villosa Roth.	a Meg-Alt T scap/ST herb	c.i.subm-sarm-pont	63,3%
FUMARIACEAE			
Fumaria officinalis L.	a Mi-Mes T scap	ev-med-subm-pont-or	53,3%

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GERANIACEAE			
Erodium cicutarium (L.) L' Hér.	v-a Mi-Mes T semiros-scap	evr (submerid-merid)	50%
Geranium columbinum L.	a Mi-Meg T scap	atl.c.ev-med-subm-pan-or	40%
Geranium dissectum L.	a Mi-Meg T scap	atl-c.ev-med-subm-pan-or	46,6%
Geranium molle L.	a Mi-mes T scap/a H scap bienn	se-sarm-med-subm	50%
Geranium pyrenaicum Burm.	a Mes-Meg H scap	atl-c.ev-med-subm	36,6%
HYPERICACEAE			
Hypericum perforatum L.	a Mes-Meg H scap	se-med-pont-j.sr.sib-or-tur	23,3%
JUNCACEAE			
Juncus articulatus L. subsp.articulatus	a Mes-Meg G rhiz caesp	evr-sam (bor-merid)	20%
LAMIACEAE			
Ajuga genevensis L.	a Mi-Mes H semiros	se-subm-pont	50%
Ajuga reptans L.	a Mes H rept	se-med-subm	33,3%
Ballota nigra L.	a Meg H scap	se-med-subm-pont-or-tur	50%
Galeopsis ladanum L.	a Mes-Meg T scap	evr (temp-submerid)-sam (sin)	43,3%
Glechoma hederacea L.	a Mes-Meg H rept/Ch herb rept	evr (subor-submerid)	50%
Lamium amplexicaule L.	v Mi-Mes T scap	evr (temp-submerid)-i.afr	70%
Lamium maculatum (L.) L.	v-a Mes-Meg H scap	se-subm-z.pont	66,6%
Lamium purpureum L.	v Mi-Mes T scap	se-med-subm-pont	63,3%
Leonurus cardiaca L.	a Meg-Alt H scap	sarm-subm-pont-j.c.sir-or-tur	23,3%
Lycopus europaeus L.	a Mes-Meg H scap/emer Hyd G rhiz	evr (subbor-merid)	26,6%
Marrubium peregrinum L.	a Meg-Alt H scap	balk-pan-z.pont	23,3%
Mentha aquatica L.	a Mes-Meg H scap	evr (subbor-submerid)-afr(boreosubtrop)	6,6%
Mentha longifolia (L.) Huds.	a Mes-Meg H scap	evr (temp-merid)-afr (boreosubtrop)	6,6%
Prunella vulgaris L.	a Mi-Mes H scap-semiros	evr-sam (subbor-submerid)	20%
Salvia amplexicaulis Lam.	a Meg-Alt H scap	i.med-i.subm	36,6%
Salvia nemorosa L.	a Mes-Meg H scap	sarm-i.subm-pont-j.sib-or-tur	30%
Salvia pratensis L.	a Mes-Meg H scap	subm-pont-j.sib	43,3%
Salvia verticillata L.	a Mes-Meg H scap	se-subm-pont-j.sib	23,3%
Scutellaria galericulata L.	a Mi-Meg G rhiz scap	evr-sam (bor-temp)	16,6%
Stachys palustris L.	a Mes-Meg H scap	evr-sam (bor-submerid)	13,3%
Teucrium chamaedrys L.	Mes Ch suff caesp	med-subm-z.pont-or	20%
Thymus longicaulis C. Presl.	v-a Mi-Mes Ch herb rept	balk (end.)	16,6%

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LILIACEAE			
Ornithogalum umbellatum L.	v Mi G bulb scap	se-med-subm-pan-z.pont	13,3%
LINACEAE			
Linum austriacum L.	a Mi-Meg H scap	subm-pan-pont-(herc)	23,3%
LYTHRACEAE			
Lythrum salicaria L. var. tomentosum (Mill.) D	a Meg-Alt H scap	kosm (evr)	16,6%
MALVACEAE			13,3%
Althaea hirsuta L.	a Mes-Meg T scap	med-subm-pan-pont-or	10%
Alcea rosea L.	a Meg-Alt H scap	adv (kult)	10%
Malva neglecta Wallr.	a Mes-Meg T scap	med-pont-or-tur	20%
Malva pusilla Sm.	a-aut Mes-Meg T scap	evr (temp-submerid)	13,3%
Malva sylvestris L.	a Meg-Alt H scap bienn/a H scap	kosm (evr)	13,3%
MORACEAE			
Maclura pomifera (Raf.) C.K. Schneid.	fo dec Mes P scap	adv (sam, kult)	3,3%
ONAGRACEAE			
Epilobium angustifolium L.	a Meg-Alt H scap	evr-sam (bor-submerid)	10%
Epilobium hirsutum L.	a Mes-Meg H scap	evr (subbor-merid)-afr (boreo-austrosubtrop)	10%
OLEACEAE			
Fraxinus ornus L.	fo dec Mes P scap	c.med-i.subm	6,6%
Ligustrum vulgare L.	fo dec NP caesp	atl-se-med-subm-or	13,3%
OROBANCHACEAE			
Melampyrum arvense L.	a Mes-Meg T scap	evr (temp-submerid)	16,6%
Rhinanthus major L.	v-a Mes-Meg T scap	se	20%
PAPAVERACEAE			
Chelidonium majus L.	v-a Mes-Meg H semiros	evr (temp-submerid)	36,6%
Papaver dubium L.	a Meg T scap	atl-se-med-subm-z.pont-or-afr (boreosubtrop)	56,6%
Papaver rhoeas L.	a Meg t scap	ev-med-subm-pont-j.sib-or	46,6%
PHYTOLACCACEAE			
Phytolacca americana L.	a-aut Alt G rhiz scap	adv (sam)	3,3%
PLANTAGINACEAE			
Plantago lanceolata L.	a Mi-Meg H ros	evr (subbor-temp)	66,6%
Plantago major L.	a Mes-Meg H ros	kosm (evr-sam)	50%
Plantago media L.	a Mes-Meg H ros	evr (temp-submerid)	46,6%

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PLATANACEAE			
Platanus orientalis L.	fo dec Mes P scap	adv (?, kult)	6,6%
POACEAE			
Achnatherum calamagrostis (L.) P. Beauv.	a Meg-Alt H scap	JEP (južnoevropsko planinska)	23,3%
Aegilops cylindrica Host	a Mi-Mes T scap	i.med-subm-pont-or-tur	43,3%
Aegilops neglecta Bertol.	v-a Mes T caesp	med-subm-tur	40%
Alopecurus myosuroides Huds.	a Mes-Meg T caesp	atl-med-subm-or-tur	30%
Alopecurus pratensis L.	a Meg-Alt H caesp	evr (subbor-submerid)	23,3%
Anisantha sterilis (L.) Nevski	a Mes-Meg T caesp	se-sarm-med-subm-pont-or	53,3%
Anisantha tectorum (L.) Nevski	a Mes-Meg T scap	ev-med-subm-pont-j.sib-or-tur	40%
Anthoxanthum odoratum L.	a Mes-Meg H caesp	ev-med-subm-z.pont-sr.sib	23,3%
Apera spica-venti (L.) P. Beauv.	a Mes-Meg T scap	ev-subm-pont-j.sr.sib	26,6%
Arrhenatherum elatius (L.) J. Presl & C. Presl	a Meg-Alt H caesp	atl-se-subm-sarm	30%
Botriochloa ishaemum (L.) Keng.	a Mes H caesp	evr (temp-merid)	13,3%
Briza media L.	a Mes-Meg H caesp	evr (subbor-submerid)	20%
Bromus arvensis L.	a Mes-Meg T scap	subm-sarm-sr.sib	50%
Bromus commutatus Schrad.	a Meg T scap	subatl-se-c.subm	46,6%
Bromus hordeaceus L.			40%
Bromus inermis (Leyss.) Holub	a Meg-Alt H caesp	evr (subbor-temp)-sam(temp-submerid)	30%
Bromus racemosus L.	v-a Mes-Meg T scap	ev-z.az	43,3%
Bromus squarrosus L.	a Mes-Meg T scap	med-subm-pan-pont-j.sib-or-tur	53,3%
Cynodon dactylon (L.) Pers.	a Mes G rhiz rept-caesp	kosm (med-or-tur)	53,3%
Dactylis glomerata L.	a Meg H caesp	ev-med-subm-pont-j.sib-or-tur-ca	30%
Dasypyrum villosum (L.) P. Candargy	a Mes-Meg T scap	med-subm	33,3%
Digitaria sanguinalis (L.) Scop.	a Mes T caesp-rept	evr-sam (subbor-merid)	23,3%
Echinochloa crus-galli (L.) P. Beauv.	a Meg-Alt T caesp	kosm (subtrop-trop)	26,6%
Eragrostis pilosa (L.) P. Beauv.	a Mes-Meg T caesp	kosm (med)	20%
Elytrigia repens (L.) Nevski	a Mes-Meg G rhiz caesp	kosm (evr)	16,6%
Festuca rubra L. subsp. rubra	a Mes-Meg H caesp	evr-sam (bor-merid)	26,6%
Festuca valesiaca Gaudin	a Meg H caesp	ev-subm-pont-j.sib-ca	20%
Glyceria fluitans (L.) R.Br.	v-aut Meg-Alt Hyd G rhiz	evr (bor-submerid)-sam	13,3%
Holcus lanatus L.	a Meg H caesp	atl-med-se-sarm-z.pont	30%
Hordeum murinum L.	a Mes T caesp	med-subm-sarm-z.pont	60%
Hordeum murinum L.	a Mes T caesp	med-subm-sarm-z.pont	53,3%

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subsp. leporinum (Link) Arcang.			
Koeleria macrantha (Lebed.) Schult.	a Mes H scap	evr-sam (temp-submerid)	13,3%
Lolium perenne L.	a Mes H caesp	ev-med-subm	56,6%
Lolium remotum Schrank	a Mes-Alt T scap	evr (temp)	53,3%
Melica ciliata L.	a Mes-Meg H caesp	se-med-subm (ev)	16,6%
Ochlopoa annua (L.) H. Scholz.	v-aut N-Mes T caesp	kosm (evr-sam)	43,3%
Pennisetum glaucum (L.) R.Br.	a-aut Mes-Meg T caesp	kosm (evr-sam)	20%
Phragmites australis (Cav.)Trin ex Steud.	Alt emer Hyd G rhiz	kosm (evr-sam)	6,6%
Poa bulbosa L. subsp.. vivipara (Koeler) Arcang.	a Mes-Meg H scap	ev-med-subm-pont-j.sib-or-tur-ca	23,3%
Poa compressa L.	a Mes H caesp	kosm (evr)	26,6%
Poa nemoralis L.	a Mes-Alt H scap	evr-sam (bor-submerid)	53,3%
Poa angustifolia L.	a Mes-Meg H caesp	kosm (evr-sam)	36,6%
Poa trivialis L.	a Mes-Meg H caesp	kosm (evr)	50%
Schenodorus arundinaceus (Schreb.) Dumort	a Meg-Alt H caesp	subm-pont-j.sib-tur	26,6%
Schenodorus pratensis (Huds) P. Beauv.	a Meg H caesp	ev-subm-pont-j.sib-ca	30%
Sclerochloa dura (L.) P. Beauv.	v N-Mi T caesp	med-subm-pont-or-tur	40%
Setaria viridis (L.) P. Beauv.	a-aut Mes-Meg T caesp	kosm (evr-sam)	36,6%
Sorghum halepense (L.) Pers.	a-aut Meg-Alt G rhiz caesp	adv (paleotrop)	33,3%
Vulpia myurus (L.) C.C.Gmel.	a Mes-Meg T caesp	se-med-subm-z.pont-or-tur-ca	13,3%
POLYGONACEAE			
Fallopia convolvulus (L.) Á. Löve	a Mes-Meg T scap/SH herb	kosm (evr)	23,3%
Fallopia dumetorum (L.) Holub	a Meg T scap/SH herb	evr (temp-submerid)	13,3%
Persicaria lapathifolia (L.) Delarbre	a-aut Meg T scap	evr (bor-trop)-sam (subbor-boreotrop)	6,6%
Persicaria maculosa Gray	a-aut Meg T scap	evr (bor-merid)	6,6%
Polygonum aviculare L.	a-aut Mi-Meg T rept	kosm (trop)	53,3%
Rumex acetosella L.	a Mes-Meg H scap	evr-sam (bor-merid)	40%
Rumex crispus L.	a Meg-Alt H scap	kosm (evr)	30%
Rumex obtusifolius L.	a Meg H scap	se-subm-pont	20%
Rumex pulcher L.	v-a Mes-meg H scap/v-a T scap	med-subm-or	36,6%
PORTULACACEAE			
Portulaca oleracea L.	a Mes T scap	adv (az)	10%
PRIMULACEAE			
Anagallis arvensis L.	v-aut Mi T rept	kosm (med)	16,6%
Anagallis foemina Mill.	v-aut Mi T rept	se-med-subm-z.pont	6,6%

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RANUNCULACEAE			
Adonis flammea Jacq.	a Mes-Meg T scap	subm-se-pan-z.pont-or	23,3%
Clematis vitalba L.	a dec S lig	se-med-subm	33,3%
Consolida orientalis (J.Gay.)Schrödinger	a Meg T scap	med-or-tur-z.pont	30%
Consolida regalis Gray	a Mes-Meg T scap	se-subm-pont-j.sib	20%
Ficaria verna Huds.	v Mi-Mes G scap	evr (bor-submerid)	16,6%
Nigella damascena L.	a Mes T scap	med-subm-or	6,6%
Ranunculus arvensis L.	a Mes-Meg T scap-semiros	se-med-or-tur-ca	36,6%
Ranunculus millefoliatus Vahl	v-a Mes H scap/G tub	c.i.med-subm	20%
Ranunculus repens L.	a Mes-Meg H rept	evr (bor-submerid)	40%
Ranunculus sardous Crantz	a Mes-Meg T scap-semiros	se-med-subm	13,3%
Ranunculus sceleratus L.	a Mes-Meg emer Hyd T semiros	kosm (evr)	16,6%
Ranunculus serbicus Vis.	a Meg H scap/G rhiz	sr.balk.j.apen (subend)	13,3%
RESEDACEAE			
Reseda lutea L.	a Mes-Meg H scap/a T scap	se-med-subm-pont-or	23,3%
Reseda luteola L.	a Mes-Alt H scap bienn/T scap	atl-se-med-subm-or	26,6%
Reseda phyteuma L.	v-a Mes-Mac T scap/H scap	med-subm	6,6%
ROSACEAE			
Agrimonia eupatoria L.	a Meg H scap	ev-med-subm-or-pont-j.sib-tur	30%
Crataegus monogyna Jacq.	fo dec NP caesp	se-med-subm-pont	3,3%
Filipendula vulgaris Moench	a Meg H scap	evr (subbor-submerid)	10%
Fragaria vesca L.	a Mes H rept	evr (subbor-submerid)-sam (temp)	16,6%
Geum urbanum L.	a Meg H scap	se-pont-j.sr.sib-tur	23,3%
Potentilla argentea L.	a Mes-Meg H scap	evr (temp-submerid)	26,6%
Potentilla hirta L.	a Mes-Meg H scap	(ev) med-subm-z.pont	26,6%
Potentilla neglecta Baumg.	a Mes-Meg H scap	evr (temp-submerid)	16,6%
Potentilla reptans L.	a Mi-Mes H rept	kosm (evr)	43,3%
Rosa canina L.	fo dec NP caesp	ev-med-subm-pont-or-tur	33,3%
Rubus caesius L.	fo dec NP rept	evr (temp-submerid)	30%
Rubus ulmifolius Schott	fo dec NP rept	atl.z.c.med-subm	20%
Sanguisorba minor Scop.	a Mes-Meg H scap	evr (temp-submerid)	50%
RUBIACEAE			
Cruciata laevipes Opiz	v-a Mes-Meg H scap	evr (temp-submerid)	36,6%
Galium aparine L.	Mes-Meg ST herb	kosm (evr)	50%
Galium lucidum All.	a Mes-Meg H scap	med-subm-se	30%

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Galium mollugo L.	a Meg-Alt H scap	se-med-subm	40%
Galium verum L.	a Mes-Meg H scap	kosm (med)	23,3%
Sherardia arvensis L.	v-a Mi-Mes T scap	kosm (med)	16,6%
SALICACEAE			
Populus alba L.	fo dec Mes P scap	se-med-subm-pont-j.sib-tur	16,6%
Populus nigra L.	fo dec Mes P scap	se-med-subm-pont-j.sib	16,6%
Salix alba L.	fo dec Mes P scap	ev-med-subm-or-pont-j.c.sib-ca	16,6%
Salix triandra L.	fo dec Mes P caesp-scap	evr (bor-submerid)	16,6%
SANTALACEAE			
Comandra umbellata (L.) Nutt.	v-a Mes-Meg Ch frut/Ch suffr	subdac	3,3%
Thesium arvense Horv.	a Mes H scap/H bienn	evr (temp-submerid)	13,3%
SAPINDACEAE			
Acer negundo L.	fo dec Mes P scap	adv (sam)	10%
Acer pseudoplatanus L.	fo dec Mes P scap	adv (sam)	10%
Acer saccharinum L.	fo dec Mes P scap	se	10%
SCROPHULARIACEAE			
Antirrhinum majus L.	Mes-Meg Ch suff caesp	adv (med-subm, kult)	6,6%
Linaria genistifolia subsp. sofiana (Velen.) Chater & D.A.Webb	a Mes-Meg H scap	balk (end)	43,3%
Linaria vulgaris Mill.	a-aut Mes-Meg H scap	evr (subbor-submerid)	23,3%
Verbascum phlomoides L.	a Meg-Alt H ros bienn	se-med (ev)-subm (ev)-pan-z.pont	56,6%
Veronica anagallis-aquatica L.	a Mes-Meg H scap	kosm (evr)	16,6%
Veronica arvensis L.	v-a N-Mes T scap	kosm (med)	20%
Veronica beccabunga L.	v-a Mes-Meg H rept	evr (bor-submerid)	23,3%
Veronica chamaedrys L.	v-a Mi-Mes H scap	se-subm-pont-j.sib	43,3%
Veronica hederifolia L.	v Mi-Mes T scap	se-med-subm-pont	30%
Veronica persica Poir.	v-aut N-Mes T scap	kosm (med-subm)	26,6%
Veronica praecox All.	v-a Mi-Mes T scap bienn	pont-subm	36,6%
Veronica serpyllifolia L.	v-aut Mi-Mes H rept	kosm (evr)	46,6%
SOLANACEAE			
Datura stramonium L.	a-aut Meg-Alt T scap	kosm (evr-sam)	23,3%
Hyoscyamus niger L.	a-aut Mes-Meg T scap/H scap bienn	evr (temp-merid)	20%
Lycium barbarum L.	fo dec NP caesp	adv (med)	3,3%
Physalis alkekengi L.	a-aut Mes-Meg G rhiz rept	se-med-subm-z.pont	3,3%
Solanum dulcamara L.	a Meg-Alt S lig	evr (temp-submerid)	23,3%

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Solanum nigrum L.	v-aut Mes-Meg T scap	kosm (evr-sam)	26,6%
TYPHACEAE			
Typha angustifolia L.	Alt emer Hyd G rhiz	evr-sam (subbor-temp)	3,3%
URTICACEAE			
Urtica dioica L.	a Mes-Alt H scap	evr-sam (bor-temp)	66,6%
Urtica urens L.	a Mes-Meg T scap	evr-sam (bor-temp)	20%
VERBENACEAE			
Verbena officinalis L.	a Mes-Meg H scap	kosm (evr-s.afr)	36,6%
VIOLACEAE			
Viola arvensis Murray	v-aut Mi-Mes T scap/a H scap bienn	evr (subbor-merid)	33,3%
Viola tricolor L.	v-a Mes T scap	evr (temp-submerid)	36,6%

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According to the literature data on vascular flora of Serbia, the families with the greatest number of species are: *Asteraceae, Poaceae, Fabaceae, Caryophyllaceae, Brassicaceae, Lamiaceae, Scrophulariaceae, Apiaceae* (Stevanović et al., 1995). In the ruderal flora of Kosovska Mitrovica and its surroundings the largest families coincide with the most numerous families in the flora of entire Serbia (*Asteraceae*-77 species, *Poaceae*-49 species, *Fabaceae*- 46 species, *Brassicaceae*- 36, *Lamiaceae*- 22 species, *Caryophyllaceae*-20 species, *Apiaceae*-16 species, *Scrophulariaceae*-12 species). The result was anticipated because of the synanthropic character of a great number of representatives of these families.

The most profuse genera found within the taxonomic floral structure in Kosovska Mitrovica area were the following: *Vicia* (11 sp.), *Trifolium* (9 sp.), *Veronica* (8 sp.), *Medicago* (7 sp.), *Bromus* and *Ranunculus* (each with 6 sp.), *Centaurea*, *Crepis*, *Sonchus*, *Silene*, *Euphorbia*, *Poa*, (each with 5 sp.), *Cirsium*, *Rorippa*, *Geranium*, *Salvia* and *Rumex* (each with 4 sp.). These genera usually include a large number of ruderal and ruderal-segetal plants. The species comprising these genera show the anthropogenic character of diverse ruderal habitats indicating either their nitrification or their xerothermic features, or the presence of intensive treading, mowing, and other anthropogenic influences that make ruderal habitats highly dynamic and instable biotopes (Jovanović, 1994).

General life form spectrum

The flora of Central European cities is dominated by hemicryptophytic life forms (Sukopp, 1990), while therophitic life forms prevail in South European city flora (Stešević et al., 2014). The ecological analysis in Kosovska Mitrovica showed the hemicryptophitic type of ruderal flora (189 sp. 42,56 % of total) (*Fig. 2)*. The dominant role of hemicryptophytes is potentially even more emphasized by the fact that among the primary annual (one year old) plants (therophytes) there are 25 species that, under specific conditions, alternate as two years old forms. The hemicryptophyte life form is largely described by trunk (stem) shapes (H scap), with 111 species. The domination of hemicryptophytes in the biological spectrum of the ruderal flora of Kosovska Mitrovica corresponds to a dominant presence of these life forms in the whole flora of the Balkans (Turill, 1929). Repeated field researches, 20 years after, showed that the number of hemicryptophytes in the ecological spectrum of life forms had increased by 19 representatives. This may be confirmed by the fact that some surfaces where the floral material was collected have ecologically stabilized.

Therophytes participated less (179 sp., 40,31% of total), which classified them as second in the biological spectrum of Kosovska Mitrovica ruderal flora. As a rule, the higher the impact of anthropogenic factor in a ruderal habitat, the greater the participation of therophytes on account of one and two years old species, which leads to the biospectrum modification (Jovanović, 1994). The total number of the newly reported or discovered plants that belong to this life form is 16. Other life forms were present with a smaller number of representatives: geophytes (25 sp., 5,63%), phanerophytes (20 sp., 4,50%), chamaephytes and scandentophytes with equal number of species (12 sp., 2,70%), and hydrophytes with 7 sp., 1,57%.

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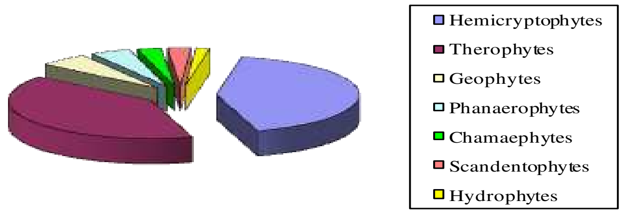

Figure 2. *Life form spectrum of ruderal flora of Kosovska Mitrovica (Serbia)*

Phytogeographical analysis of ruderal flora

Ruderal flora, as a specific plant category, is under the anthropogenic impact. It is unsuitable for the analysis whose aim is to determine floral and geographical belonging to certain area (Jovanović, 1985). This could be explained by the fact that ruderal flora encompasses species of vast areas whose diffusion was (directly or indirectly) influenced by man. There is also a fact that the composition and diversity /abundance of ruderal flora and vegetation of some area are proportional to the intensity of anthropogenic influences and may serve as an indicator of environmental quality and urbanization level. A detailed phytogeographic analysis of Kosovska Mitrovica ruderal flora corroborated the presence of 7 basic area types that incorporate 18 areal groups *(Fig. 3).*

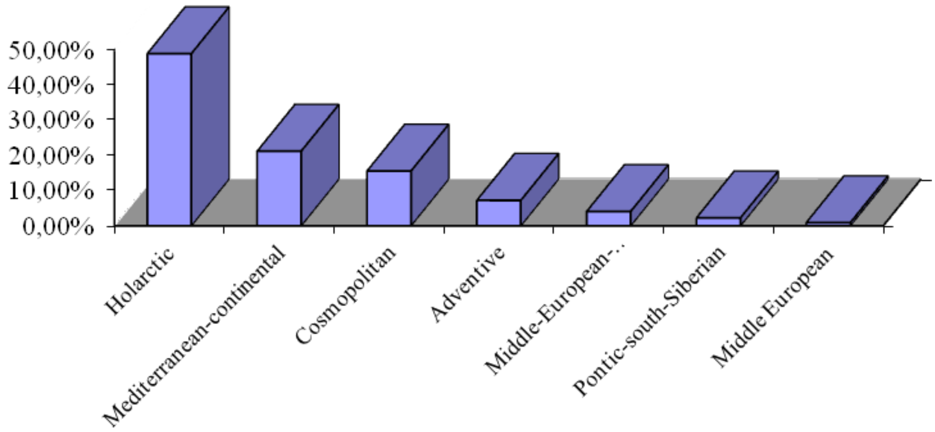

Figure 3. *Chorological spectrum, by basic area types, of the entire ruderal flora of Kosovska Mitrovica (Serbia)*

Most numerous species were of the Holarctic area type, presented with 217 species or 48,87%, within which the European, Western-Asian and Euroasian group of floristic elements dominated. According to its number, Mediterranean-continental type is

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second, with 94 taxa (21,17%) united in 4 different groups of area floral elements. The areal types of Cosmopolitan species are represented by 69 taxa (15,54%). The cosmopolits of Euroasian and Mediterranean origin are larger in number compared to the Cosmopolits of circumholarctic and tropical origin.

The presence of adventive plant species is relatively high (32 taxa or 7,21%). The increased number of adventive and cosmopolitan species shows the instability of urban and suburban habitats, because 19 new taxa have been reported in the past 20 years (from the total of 58 recently recorded) and they all belong to the given area type. The common representatives are: *Symphyotrichum novi-belgii* (L.) G.L.Nesom, *Symphyotrichum salignum* (Willd.) G.L.Nesom, *Calendula officinalis* L., *Helianthus tuberosus* L., *Solidago gigantea* Aiton, *Phytolacca americana* L., *Antirrhinum majus* L., etc. It could be noted that they are the species that man brings into the urban habitats, mostly as decorative and horticulture plants. Due to their pronounced biological potential they conquer new habitats and anthropogenically spread from urban areas to natural (uncultivated) surroundings, usually after removing the plant remnants in autumn and placing them in landfills /disposal sites. The least reported species in the ruderal flora of Kosovska Mitrovica is Middle-European-Mediterranean areal type (18 species), Ponto-South Siberian (10 species) and Middle European dispersion areal type (4 species).

Ecological analysis of ruderal flora

The contemporary way of life and urbanization (demolition and new construction, changes in land use, traffic, etc.) in the town have resulted in fragmentation of habitats or their complete loss. Rapid urbanisation in Kosovska Mitrovica over the last decade has caused a visible decrease in green areas, but also the change of floral composition of the ruderal flora. Following the dynamics of appearance of the found ruderal floras in certain localities, it is possible to ascertain their ecological (phenotypic) plasticity and their adaptability to various conditions that prevail on investigated surfaces. The most widespread species *Convolvulus arvensis* is the most prevailing species (93,3%) in 28 out of 30 researched areas. The given species inhabits sunny and open ruderal habitats where it vies with other species for nutrients and available humidity. It also may decrease habitat biodiversity. It is one of the most serious weeds of agricultural fields in temperate regions of the world.

According to its prevalence degree of 76,6% and phenotypic plasticity and adaptability, *Taraxacum officinale* occupies the second place and inhabits the most diverse ruderal habitats, which may be confirmed by the results of the comparative morpho-anatomical research of the species *T. officinale* whose samples originate from trodden and untrodden areas of Belgrade (Stevanović et al., 1988). From the ecoclimatic point of view, the trodden ruderal habitats along with the majority of other types of ruderal habitats, may be defined as xerothermic with a favourable regime but with weak soil aeration and severe water deficiency. *Taraxacum officinale* has very large leaves with mesomorphic structure on untrodden surfaces. The members of this species on trodden areas show a variety of xeromorphic features. This indicates a significant phenotypic plasticity of the species and their adaptability to the changed environments in different ruderal habitats.

Species that were found in more than 50% of the sampling sites were: *Plantago lanceolata, Capsella bursa-pastoris, Cardaria draba, Lactuca serriola, Lotus corniculatus* (prevalence= 66,66%), *Artemisia vulgaris, Stellaria media, Chenopodium*

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album, Melilotus officinalis, Achillea millefolium (prevalence=60%), *Polygonum aviculare, Medicago lupulina, Cynodon dactylon, Anisantha sterilis, Poa nemoralis, Hordeum murinum subsp. leporinum* (prevalence=53,3%). The group of eurivalent species with 50% of frequency includes: *Silene vulgaris, Tragopogon dubius, Trifolium repens, Tripleurospermum inodorum , Erodium cicutarium, Galium aparine, Cichorium intybus and Ballota nigra* . The species like *Convolvulus arvense, Taraxacum officinale, Polygonum aviculare*, *Cynodon dactylon* are prominent due to their exceptionally pioneering character and the ability to adapt to diverse, often extreme and difficult conditions of ruderal habitats.

Diametrically opposed to the group of highly frequent ruderal species is a number of species with a limited range-stenovalent plants (prevalence=6,6% or 3,3%), which , in accordance with their ecology, develop only in certain ruderal habitats. The group of stenovalent plants is represented by ruderal higrophile species that appear on nitrified, humid and periodically flooded river banks, such as: *Bidens tripartitus, Persicaria maculosa, Persicaria lapathifolia, Mentha aquatica, Mentha longifolia* etc. This group may also include species such as: *Comandra elegans, Asparagus tenuifolius* and they appear only in one investigated area, as the remains of the primary vegetation form. We assume that, as the anthropogenic influence grows and intensifies in the mentioned localities, some of these species will become extinct because the ruderal habitats do not suit them, i.e. they do not provide ecological optimum for their development.

Sukopp (1973) presents data that between 1850 – 1950 some European cities faced the loss of flora by 4-16%. Klotz (1987) and Landolt (2000) show that the number of species in the cities flora has remained approximately the same in the past 1-2 centuries, but the flora composition has changed by 30- 40% and provided an advantage for the adventive species (Stešević et al., 2014). Our analyses show that the ruderal flora composition in Kosovska Mitrovica area has changed by 13.06% in the past 20 years. It is also noted that there has not been a reduction in a total number of species. Autochthonous species were replaced by allochthonous-cosmopolitan species,which as more adaptable to homogenous and degraded urban habitats, are beginning to compete with natural species and are gradually ousting them.

The introduced species that might become invasive represent a special menace and may cause destruction of natural ecosystems and changes in their biodiversity. Newly introduced plants most frequently inhabit ruderal habitats that are temporarily or permanently under the anthropogenic influence. The most widely accepted definition of invasive species is the one advocated by the global environmental organisation IUCN (International Union for Conservation of Nature): ”*An invasive alien species (IAS) is a species that is established outside of its natural past or present distribution, whose introduction and/or spread threaten biological diversity*” Convention on Biological Diversity. Out of total number, 38 plant species from the weed ruderal flora of Kosovska Mitrovica and its surroundings, are labelled as invasive (*Acer negundo, Ambrosia artemisifolia, Symphyotrichum novi-belgii, Symphyotrichum salignum, Erigeron canadensis, Galinsoga parviflora, Helianthus tuberosus, Solidago gigantea, Xanthium orientale subsp. italicum, Xanthium spinosum, Centaurea solstitialis, Artemisia vulgaris, Cichorium intybus, Amaranthus albus, Amaranthus blitoides, Amaranthus retroflexus, Bifora radians, Armoracia rusticana, Coronopus squamatus, Echinocystis lobata, Chenopodium strictum, Amorpha fruticosa, Medicago sativa, Robinia pseudoacacia, Alceae rosea, Echiochloa crus-galli, Sorghum halepense, Dasypyrum villosum, Phytolacca americana, Polygonum aviculare, Portulaca*

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oleraceae, Consolida orientalis, Consolida regalis, Rubus caesius, Veronica persica, Datura stramonium, Lycium barbarum, Urtica dioica). The largest number of their representatives belong to *Asteraceae family* (12 species), followed by *Amaranthaceae, Fabaceae* and *Poaceae* (each with 3 species). The investigations show that there has been an increase of invasive species and they have become more frequent in every investigated locality over the past 20 years.

If we analyse the origin of invasive species within weed ruderal flora of Kosovska Mitrovica and its surroundings, we may come to the conclusion that the species originating from North American continent (19 of them-50%) prevail in number. These species were mostly introduced as decorative plants (*Robinia pseudoacacia, Acer negundo*), but they soon "escaped" to the wilderness attacking the natural ecosystems with detrimental effects and became invasive. It is interesting to mention the *Ambrosia artemisfolia* species unreported in Kosovska Mitrovica and its surroundings until 2012 has been randomly spotted on certain ruderal places in the city core ever since.

Currently, the larger part of the society perceive ruderal urban plants as common weeds and do not appreciate their stability and aesthetic value. Currently the aesthetic and ecological values of such plant communities are indiscernible and underestimated. However, colourful flowering plant communities in cities have a positive influence on human senses (Kazimierska et al., 2009). Except in the case of allergenic plants and their negative influence on human health, it can be generally stated that the flora and vegetation of man-made environments represent a significant, spontaneously developed phytohealing factor, the one which in such polluted conditions produces a whole series of positive effects so they deserve a better treatment (Stešević and Jovanović, 2008).

In an industrial city, such as Kosovska Mitrovica, a spontaneously developed plant cover has undoubtedly many positive effects (oxygen production, carbon-dioxide assimilation, soil overheating avoidance, soil erosion prevention, esthetic and health-hygienic protection of the unhygienic areas, decoration) so it should be "exploited" and adjusted to fit human needs (which implies prevention of undesirable species spreading, their blooming and fruiting) and organize a monitoring process over an extended period of time to evaluate the changes in habitat conditions, as well as in floristic composition.

Conclusion

Due to expected and intense anthropogenic activity on ruderal flora in Kosovska Mitrovica and its surroundings, there was a change in overall floral composition by 13,06%, over a period of 20 years (1996-2016). A total of 444 taxa of vascular plants classified into 271 genera and 58 families were found in various types of ruderal habitats; with 58 new taxa reported in recent field explorations. An analysis of life forms in the ruderal flora of Kosovska Mitrovica showed that after a period of 20 years, the investigated area still has hemicryptophyto-therophyte character, with domination of hemicryptophytes. Phytogeographic analysis revealed the presence of 7 basic area types that incorporate 18 areal groups. The most numerous species were of the Holarctic area type, presented with 217 species or 48,87%. The increased number of adventive and cosmopolitan species shows the instability of urban and suburban habitats, because 19 new taxa have been reported in the past 20 years (from the total of 58 recently recorded) and they all belong to the given area type. The most prevailing species are

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Convolvulus arvense and *Taraxacum officinale*. Out of total number of recorded species, 38 are labelled as invasive.

All botanical studies should definitely include monitoring biodiversity of urban flora and new short term researches. It is the only method to identify potential threats to autochtonous flora and to enable a timely anthropogenous action to curb spreading of invasive, potentially allergenic species as well as aggressive, competitive species that might have negative effects on human population and urban habitats.

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A SIMPLE METHOD FOR DETERMINATION AND CHARACTERIZATION OF IMIDAZOLINONE HERBICIDE (IMAZAPYR/IMAZAPIC) RESIDUES IN CLEARFIELD® RICE SOIL

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Abstract. A study was conducted to evaluate residues of imidazolinone (IMI) in soil. Samples were taken from three Clearfield® rice fields as IMI which have been used for six years. IMI herbicides (imazapic/imazapyr) were widely used in Clearfield® rice soils. To date, few studies are available on the residues of these herbicides, especially in the context of Malaysian soil. Therefore, for this purpose, high performance liquid chromatography (HPLC) with UV detection was performed using a Zorbax stable bond C_{18} (4.6 × 250 mm, 5 µm) column, with two mobile phases. The average percentage recovery for imazapyr and imazapic varied from 76%-107% and 71-77%, with 0.1-5 µg/ml fortification level, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.05 and 4.09 for imazapic and 0.171 and 0.511 µg/ml for imazapyr respectively, in the top 15 cm. In the extracted soil sample, it was 0.19 µg/ml for imazapic and 0.04 µg/ml for imazapyr, respectively. Based on this study, a pre-harvest period of 40-60 day is suggested for rice crops after IMI application.
Keywords: *recovery, acetonitrile, Zorbax stable bond C_{18}, HPLC, terminal residues, Clearfield® rice*

Introduction

Weedy rice (WR) is a notorious weed associated with rice paddy crops. WR in Malaysia (locally known as Padi Angin) was first observed and reported in 1988 (Watanabe et al., 1996). WR also always acted as dominant competitor in terms of inter- and intra- varietal competitions among rice species (Baki and Shakirin, 2010). The application of pesticides in the agricultural system becomes unavoidable in the present day, as it increases production and decreases yield loss caused by pests (Schreiber et al., 2017). In 2010, the introduction of a new type of imidazolinone herbicide (IMI) known as OnDuty® with its main ingredient being imazapyr/imazapic, was used in Malaysia's agricultural system (Azmi et al., 2011). Because sometimes mixing two or more herbicides into one spray solution can offer producers multiple benefits (Fish et al.,

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2015). This resulted in an overnight success in rice cultivation in terms of yields and WR control. It also helps control a broad spectrum of weeds, encompassing grasses, and cyperaceous and broad-leaved plants, and those that WR are closely associated to (Neto et al., 2017).

IMI inhibits the enzyme function in the plant known as acetohydroxyacid synthase (AHAS) (Bailey and Wilcut, 2003), which starves the plant and lead to its death. Upon the introduction of IMI – resistant cultivated rice (Clearfield®), most difficulties faced by farmers that are related to WR have almost been completely solved. OnDuty® herbicide formulation containing imazapyr is a generic name for [2-(4-isopropyl- 4-methyl-5-oxo-2-imidazoline-2-yl) nicotinic acid]; trade names of Arsenal and Chopper), while imazapic is the generic name of [2-(4,5dihydro-4-methy-4-(1-methylethyl)-5-oxo-1Himidazol-2-yl)5-methyl-3-pyridinecarboxylic acid]; trade names of Cadre and Plateau (Azmi et al., 2012). Both are widely used in WR, and its efficiency has been proven. IMI is relatively persistent in soil, with half-lives ranging from 30 - 150 days (Kemmerich et al., 2015). Shorter half-lives of imazapyr (34–65 days) detected in forest soils (Michael and Neary, 1993).

IMI have two enantiomers (Krieger, 2001) derived from the chiral center, which consists of imazapyr, imazapic, imazaquin, imazamox, imazethapyr and imazamethabenz-methyl, as per *Table 1*. IMI detection and research separation from water and soil are limited compared to other types of herbicides due to the low application rate (100-200g/ha) and co-extraction of other substances that interfere with chromatogram and low rates of application (Ramezani et al., 2009; Andreu and Picó, 2004). However, In the last decade, diverse studies were done on these types of herbicides to assess the risk assessment to the environments as water and soil (Silva et al., 2009; Anastassiades et al., 2003; Andreu and Picó, 2004; Börjesson et al., 2004; Bajrai et al., 2017). Also, the massive use of IMI herbicides might have contributed to the increase in resistant weedy rice, also, in most cases, herbicides-resistant weeds go undetected until they represent about 30% of the total population (Burgos et al., 2014). Many reports showed the imidazolinone carryover affected many non-rice crops in rotational systems (Alister and Kogan, 2005).

Studies on how much of these herbicides run-off into soil and water are important due to their potentially harmful influence on the environment (Schreiber et al., 2017). Developing methods for extraction in water and soil are very important, because some studies revealed that the residues of these herbicides remain present in Swedish soil after 8 hours (Börjesson et al., 2004). Herbicidal residue is defined as the remaining herbicides on or in the soil after its application in agricultural soil. Its persistence in soil sometimes causes injuries in the next crop (Assalin et al., 2014). Most previous studies on this type of herbicides used alkaline/acidified water, methanol, acetonitrile, and diverse techniques for the extraction process (Helling and Doherty, 1995; Ramezani et al., 2009). The extraction of IMI herbicides and determination using HPLC-UV is popular in literature, and it was used by many researchers because it provides clearer and more realistic results (Pace et al., 1999; Helling and Doherty, 1995; Laganà et al., 1998). Generally, herbicidal residues are usually concentrated in the top of 10 cm, although it could leach deeper. These residues are injurious to non-tolerant rotational crops, such as wheat and corn (Alister and Kogan, 2005). Areas such as Tanjung Karang sees intensive use of IMI herbicides by farmers. The rice farmers have been applying IMI herbicides for ~6 years. We present a simple HPLC method that can be used to detect, analyze, and evaluate the residues of the imazapic and imazapyr in soils

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cultivated in Clearfield system. Reversed – phase liquid chromatography (RP-LC) with ultraviolet (UV) detection is widely used because it can separate high or medium polarity pesticides and detect them at low levels, making it one of the most powerful technique in separation methods (Hogendoorn, 2006). The farmers in this area have been applying IMI herbicides repeatedly (Mazlan et al., 2016). Therefore, this study was undertaken in the Clearfield fields to determine residues of IMI in the soil.

Material and methods

Study site description

Sawah Sempadan-Tanjung Karang district is located on (N 3°25'35.0724", E 101°10'36.1704") in Kuala Selangor, Malaysia as shown in (*Fig. 1*). The soils samples were collected on November 2016/2017 from these fields, because the farmers there have been using IMI herbicides since its introduction in Malaysia in 2010 (Azmi et al., 2012). This area is the most prosperous agricultural district in Malaysia, and has many hectares of paddy rice (Mazlan et al., 2016).To determine the final IMI residue, the soil samples were collected at harvest time, which was ~90 days after IMI was sprayed on Clearfield rice crop.

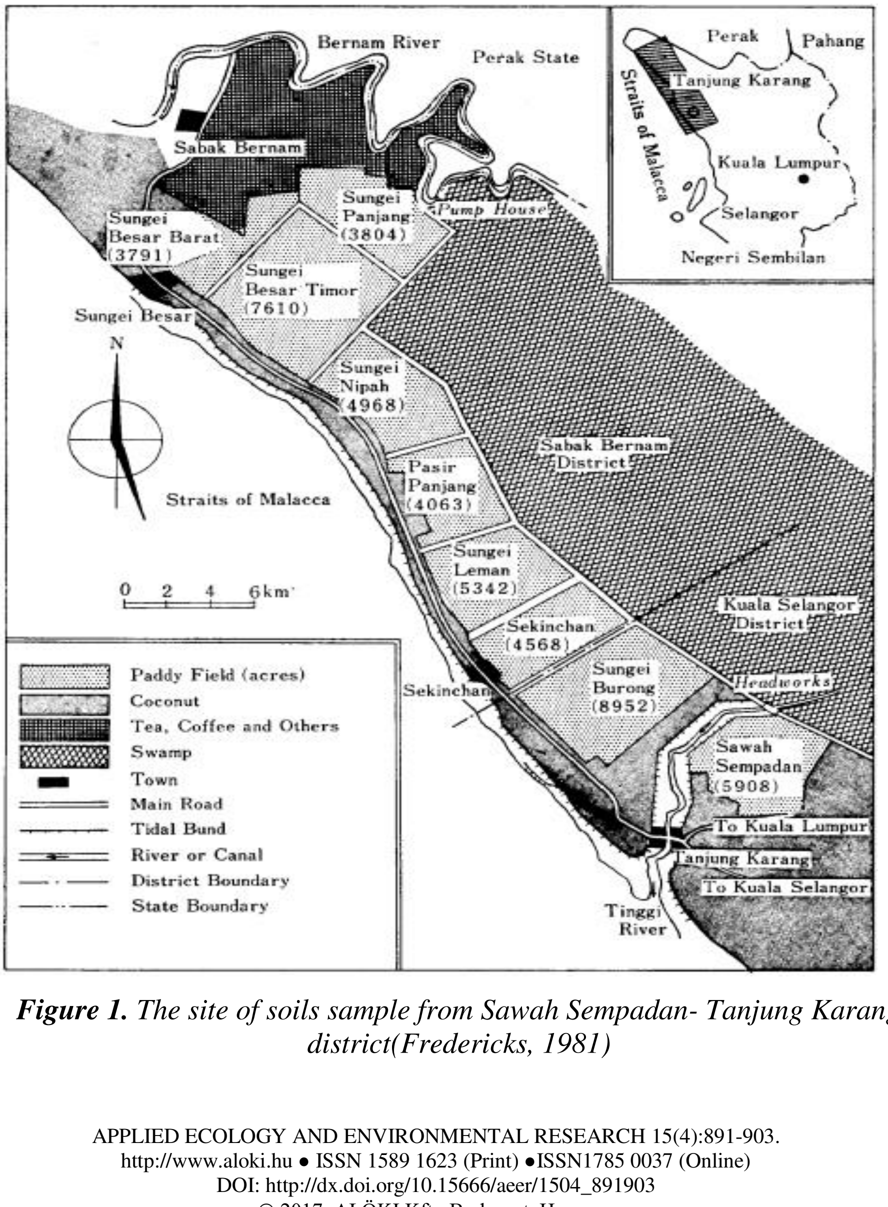

Figure 1. *The site of soils sample from Sawah Sempadan- Tanjung Karang district(Fredericks, 1981)*

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Chemicals, reagents, and apparatus

Standards of imazapyr (99.5% purity) and imazapic (99.9% purity) were purchased from Sigma-Aldrich (USA), while formic acid (85%), methanol 99.9 % (HPLC grade) and acetonitrile 99.9 % (HPLC grade), acetic acid-ACS reagent (Fisher), formic acid-98% (EM Science), Sodium phosphate (Fisher), hydrochloric acid 6N (Fisher), phosphorous acid dichloromethane (DCM) 99.9 % (HPLC grade), and Rotary evaporator were purchased from Sigma Aldrich (Germany). Ultrapure water was obtained from a Milli-Q Direct UV3® system (Millipore, USA), and was further purified by passing it through a 0.2µm Whatman filter paper. The HPLC 1100 series fitted with a UV detector was used. The HPLC column used in this work was a Zorbax RX–C_{18} (4.6 × 250 mm, 5 µm). Its temperature was maintained at 30 °C. Centrifuge-Dupont Sorvall Model RC-5C, centrifuge bottles with cap 45 ml polypropylene (Kontes Scientific), vortex mixer (Labmart 3000), Thermo-ultra-sonic, analytical balances (AUW-220D and UX-420H from Shimadzu, Japan), 0.22 µm nylon filters, glass vials with capacity of 2 mL (Agilent, USA), and screw-capped polypropylene tubes (45 ml, Germany), DSC-18 6 ml tubes 500 mg (6 cm × 3cm) SPE cartridges (supelco), anhydrous sodium sulfate, and a vacuum pump were all used in this work as well.

Stock solution and working standards preparation

Standards stock solutions of the herbicides imazapyr and imazapic were individually prepared in methanol at concentrations of (100 µg/ml), respectively, from (1000 µg/ml). Different fresh diluted solutions were prepared as 0.1, 0.5, 1, 5, 10, and 20 µg /ml, and diluted in methanol. All stock and working solutions were stored at -18 ºC in the dark (Marcia, 2014). Then, each of these solutions was injected (17 µL) into the HPLC system, at 251 nm, and peak areas were recorded and plotted versus the concentration of the herbicides.

Soil collection and preparation

The sample preparation process which involves extracting the analyte is very important and crucial. To determine the herbicidal residues from the soil samples, the samples were taken systemically from a randomly chosen area from three Clearfield® rice fields that were exposed to the herbicides. The basic approach is to analyze the depth intervals of the soils samples for each field. Each sample was within 0 - 20 cm and 20 – 40 cm, about 30-m distance between each two samples was taken with the helical shape method. A 20 soil samples were taken, and ~500-gram (gm) soil samples were collected using special auger for collection of the soil samples for increased control, and were stored in sterile zip lock polyethylene bags and coded with special code water proof stickers.

Three random samples were selected (two from each field), then one sample was selected randomly for examination, while the rest were stored in a refrigerator at a suitable temperature for subsequent analyses. The samples were air-dried in the special room at 35 C° for up to 5 days, grounded with a mortar and electronic machine, sieved through stainless steel sieve (2.0 mm) and stored at 4 °C. A100 g of homogenized soil samples was stored in polyethylene bag at a temperature of ~15 C° until it was analyzed for herbicidal residues. The soil physico-chemical characteristics were analyzed and the basic properties of these soils are shown in (*Table 1*).

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Table 1. *soil texture characteristics of three locations soil*

Location	Depth (cm)	PH	Moisture%	Sand%	Silt%	Clay%	OM%	Soil type
Field A	0-20	6.21	38	39	29	30	2	Clay loamy
	20-40	6.7	33					
Field B	0-20	6.81	44	24.6	35.7	39.2	1.3	Clay loamy
	20-40	6.61	57					
Field C	0-20	7.1	38	25	35	38	1.9	Clay loamy
	20-40	6.94	59					

Soil extraction procedure for IMI Residue level

Analyses of the samples of soil were carried out using the modified extracted published methods proposed by (Ramezani et al., 2009; Krynitsky et al., 1999). About 5 ±0.001 g of a randomly homogenized soil sample was weighted, which provides appropriate and representative amount as some authors have used (Martins et al., 2014). The portion soil was placed in 250 ml centrifuge tube (polypropylene), 150 ml of extracted 0.5 N NaOH. Then sample was kept 45 minutes in an end-over-end shaker at 30°C to assess the homogeneity of the sample. A 10 ml methanol was added to precipitate humic acids and sonicated for 10 minutes, then centrifuge the sample for 10 minutes at 7000 rpm to remove particulates.

The sample solution was filtered and adjusted to pH 2 by addition of 6N hydrochloric acid. Clean-up is necessary to shift down the detection limits of methods and to avoid interferences from the matrix. The suspension was left at room temperature for 10 minutes until analysis, then transferred into a 500 ml separatory funnel and extracted with two 50 ml portions of dichloromethane (DCM). The extracts were combined and the DCM was dried using anhydrous Na_2SO_4, then passed through smooth activated charcoal. The resulting solution was then transferred into a 250-ml round bottom flask and solvent was evaporated at 65 °C using a rotary evaporator at a low speed to near dryness. The residue was diluted with about 2 ml of a 1:1 solution of methanol:0.1% formic acid, then loaded (under vacuum) into the 6 ml DSC-18 (Supelco) solid phase extraction cartridge containing 500 mg of polymeric of adsorbing material conditioned with 3ml of each of the solvents methanol, acetonitrile, and H_2O.

The vacuum was slowly reduced and the analytes were washed with 9 ml H_2O and 6 ml (60:40) (H_2O: acetonitrile). Finally, the vials were placed in the vacuum apparatus and the cartridge eluted with 3 ml of the methanol:0.1% formic acid solution. The resulting extract was filtered through a 0.22µm polytetrafluoroethylene (PTFE) membrane, transferred to a 1.5 ml HPLC auto sampler vial, and stored at 4°C until separation by HPLC.

Method application

In this study, 20 samples were taken from the same three sites of Sawah Sempadan-Tanjung Karang district, and different chemical compositions and pH values were analyzed.

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Accuracy (%Recovery), limit of detection (LOD), and limit of Quantitation (LOQ)

LOD is the lowest concentration that can be detected, and it could be determined by a statistical method. This could be achieved by measuring the more dilute concentrations of analyte. These concentrations are expected to produce a response of ~3 times the background noise. LOD should be between 3 - 10. The LOQ is expected to behave similarly, but with a ratio of 10 times the background noise. Recovery studies in soils samples were conducted using the standard calibration curves equation.

These herbicides were spiked to blank soils (clean soils free from herbicides), taken from the land around University Malaya (N 3°7'8.9328" E101°39'28.494"). This soil was selected due to its similar characteristics with the tested soil samples. Acetone was added to 5 g of dried homogenized soil at different concentrations, and left to dry for 48h at room temperature to activate the introgression and equilibrium while slowly evaporating the solvent (Rebelo et al., 2016; Laganà et al., 2000), followed by extraction and analysis using HPLC-UV.

Results and discussion

Contamination of environmental resources by herbicides is an increasing environmental concern. Undoubtedly, soil plays a significant role in an agro-ecosystem, but information for analysis of these types of herbicide residues in the soil can be very difficult to achieve. HPLC with UV detection was chosen due to it being a fast and effective separation method. This study involves trying different columns and mobile phases for the HPLC technique. Finally, in this method a proper separation was achieved using the gradient mobile phase and C_{18} column (4.6 × 250 mm, 5 µm) was used for stationary phase separation.

Purified water was used as one of the mobile phases, due to its low cost, lack of toxicity to the environment (Laganà et al., 2000). The mobile phase acetonitrile (100%), as one of mobile phase, is the best mobile phase (Martins et al., 2014; Demoliner et al., 2010), along with purified water acidified with 10% acetic acid (pH to 2.8), due to the pH's effect on the peak shape (Singh, 2013).

Therefore, acetonitrile was chosen due to it is great solubility and higher elution strength than dichloromethane for fractionating the analytes. Acetonitrile is the best choice for the mobile phase (Singh, 2013). However, analysis was carried out using gradient solvent program using mobile phase A (acetonitrile (100%)) and mobile B (purified water acidified with 10% acetic acid (pH adjusted to 2.8). The initial gradient program was 35% A, maintained for a minute, then increased to 45% for 3 min, then decreased to 35% at 8 mins. The column temperature was set to 30C°. The flow rate was 1 ml/min, injection volume was set to 17 µL, and UV detection was set to a wavelength of 251 nm.

Simultaneously, methanol was evaporated before the sample is injected into the HPLC apparatus. Standard curve linearity and calibration was determined at six concentrations (0.1, 0.5, 1, 5, 10, and 20 µg /ml), and were prepared in the laboratory by diluting the stock solution and plotting the analytes' concentration against peak area. Each level of the concentration was analyzed repeatedly. The equation of analytical calibration was obtained by plotting the peak areas on y-axis and the concentration on the x-axis within the previous calibration levels for both imazapic and imazapyr. The concentration of both herbicides was calculated by comparing the peak values in the calibration, using the regression equation. The linearity of the method was determined from the correlation coefficient, as per *Fig. 2*.

The matrix effect has been mentioned in literature and is explained via multiple perspectives, with some reporting a shift of over 10% on the analytical results (Kemmerich et

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al., 2015). However, some that are less than 20% does not affect the matrix (Ferrer et al., 2011). The chemical analysis of these herbicides in soil are often problematic due to the low detection limits required and the pH adjustment during the extraction process. IMI is a weak acid, as per (*Table 2*), therefore their presence in soil is influenced by pH (Schreiber et al., 2017). Soil particles were fine-grinded to increase the interaction between the solvents and soil particles, which lead to increased herbicides extraction.

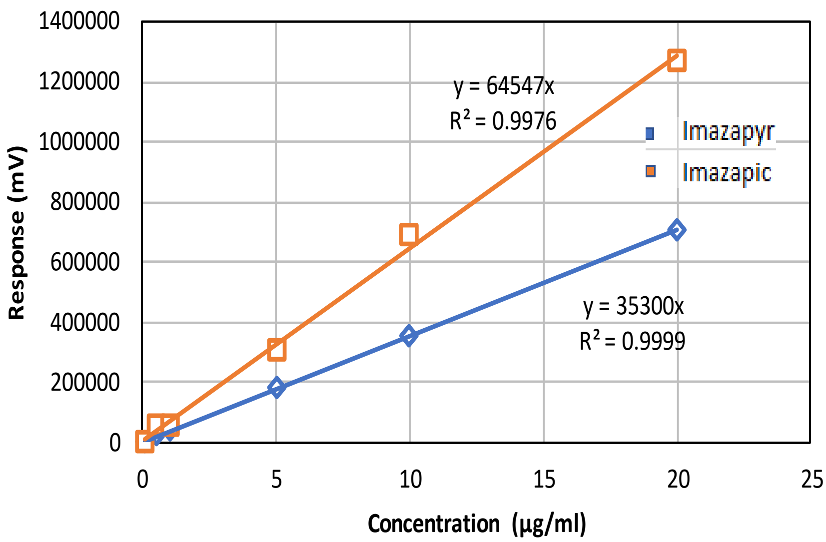
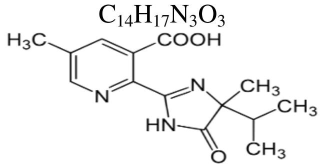
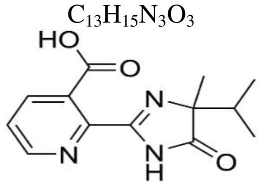


***Figure 2.** Representative calibration curve for IMI was obtained by the determination of six levels in duplicate at ranged from 0.1-20µg/ml*

***Table 2.** The characteristics (Molecular and physicochemical) of imazapic and imazapyr*

Name	[a] Imazapic	[a] Imazapyr
Family/chemical class	Imidazolinone	Imidazolinone
Trade name	Cadre, panoramic, plateou	Arsenal, Chopper, Habitat, Stalker
Chemical name	[2-(4,5dihydro-4-methy-4-(1-methylethyl)-5-oxo-1Himidazol-2-yl) 5-methyl-3-pyridinecarboxylic acid]	[2-(4-isopropyl- 4- methyl-5-oxo-2-imidazoline-2-yl) nicotinic acid]
Molecular weight	275.30308g/mol	261.2765g/mol
Molecular formula	$C_{14}H_{17}N_3O_3$	$C_{13}H_{15}N_3O_3$
Structural formula		
Water solubility	2200mg/L	9740 mg/L
Life time in soil	Around 120days	90-120 days
[b] pKa	2.1, 3.9	1.9, 3.6
[c] Goss	High potential	High potential

[a] Data quoted from (Senseman, 2007, Schreiber et al., 2017).
[b] Indicates the pH value at which 50% of total molecules are associated in soil and 50% of total molecules are dissociated.
[c] Method of classification of potential surface water contamination.

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The traditional types of extractions ordinarily use the chemical compound PSA (primary secondary amine), and due to the fact that the IMI family are present in multiple forms, it acts as a weak acid/base, which allows PSA to hold over acidic herbicides (Marcia, 2014). One of the important effects occurs when the types of herbicides have pK_a values in the range 1.3-3.9 (Krieger, 2001), which includes the weak acid IMI herbicides. Based on this, the shape of the peak area during analysis was expected to be affected by the value of the pH of the mobile phase. Soil pH and the microbial activity are the main factors in the degradation process of IMI herbicides in the soil (Sondhia et al., 2015). For example, when the pH increases, the adsorption and persistence decreases.

Also, another important factor that control the residues' concentration is the depth and type of soil. IMI sorption is correlated and increased with clay content, due to increased binding of the herbicide to soil particles, where (Gianelli et al., 2014). (Burnside et al., 1963) show that some herbicides can leach deep into the soil. For example, some studies revealed that the sorption of these types of IMI as imazapyr to sandy soils is very weak compared to its sorption to clay and humic soils (Lode and Meyer, 1999). The agricultural soils contain numerous impurities and old chemicals, which can persist for a long time, which would cause separation problems in the column, especially if the soil contained only very low concentrations of imazapic or imazapyr. Imazapyr and imazapic have the potential to leach into groundwater due to its persistence and mobility in soils, and very low volatility (Gianelli et al., 2014). Certified imazapic and imazapyr (USA) were used for calibration (*Fig. 3* and *Fig. 4*).

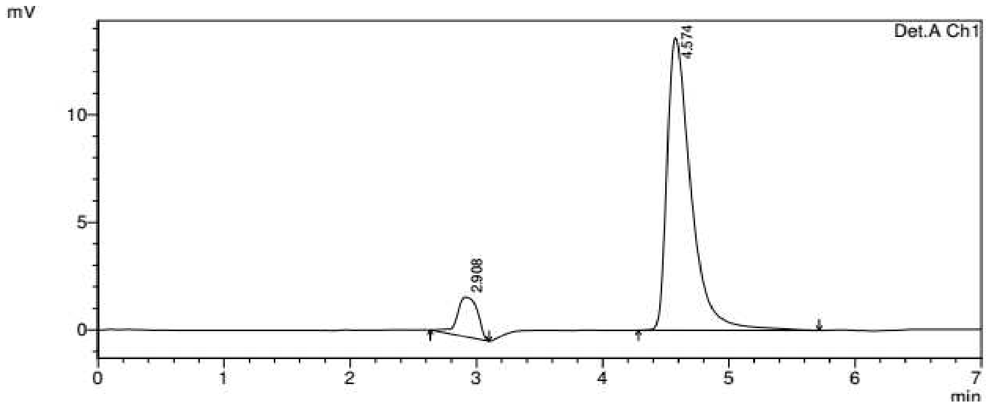

Figure 3. *Imazapyr standard, 10ppm*

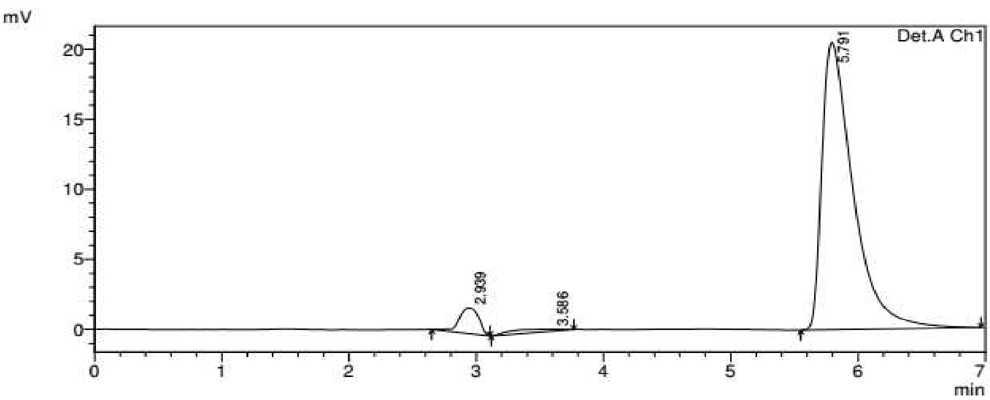

Figure 4. *Imazapic standard, 10ppm*

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The adsorption of IMI herbicides decreases by increasing heavy rain and temperatures. The higher solubility of these types in water, high temperatures, and great rainfall in Malaysia are main factors that play important roles in the transition of residual particles of herbicides via its pores or movement to other places and shift up the degradation mechanism, as per (Grey et al., 2012; Fish et al., 2015). Malaysia has almost daily high intensity rain fall and temperatures. Studies revealed that temperatures between 35C°- 45C° and increased soil moisture enhance both the chemical and microbial degradation for herbicides (Neto et al., 2017). Different methods are applicable for extraction of IMI herbicides from soil samples, but most are not satisfactory (de Oliveira Arias et al., 2014). Despite the fact that imazapyr and imazapic were applied in low doses, both can remain for long periods of time in the soil , which can cause agronomic and environmental problems (Kraemer et al., 2009). However, leaching is influenced by the environment, which means that when the water content decrease from the upper surface, it leads to increased pH. Also, some chemical herbicides move to the upper surface of the soil due to capillary action, which causes it to evaporate (Mangels, 1991).

Selectivity

Selectivity is defined as the evaluation or detection of the analyte from others analytes and different compounds that could be present at the same moment in the matrix or the sample (Ahuja, 1989). There were no matrix peaks in the chromatogram analysis that interfere with analysis of the residues as shown in *Fig. 5* and *Fig. 6*.

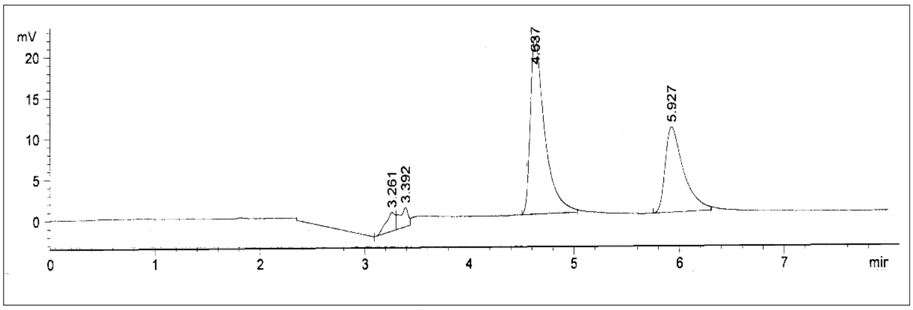

Figure 5. *Extraction imazapic and imazapyr with good resolution*

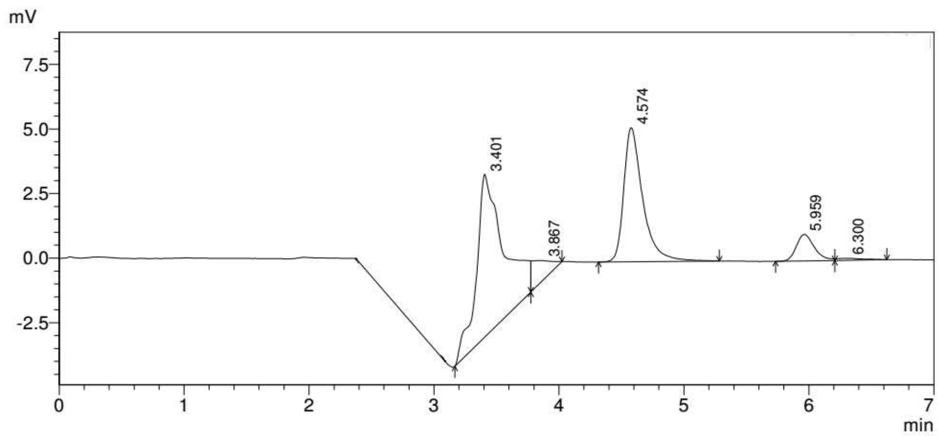

Figure 6. *Extraction imazapic and imazapyr with good resolution*

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Accuracy (%Recovery)-Limit of detection (LOD) and limit of Quantitation (LOQ)

The achieved results revealed an excellent linearity at different concentrations of imazapyr and imazapic standards in the range from 0.1 to 5 µg/mL. These herbicides' concentrations are spiked to blank soils as described in the experimental section.

Due to the spiking of the extracts, the final comparison between the two systems is expected to be valid. The precision and recovery for the two herbicides was calculated through the injection of freshly prepared six standards. The proportion of the area of the peak of herbicide resulting from the spiked solution to the area of the herbicide peak resulting from a standard solution prepared previously was calculated. The average percentage recoveries for imazapyr and for imazapic varied from 76%-107% and 71-79% with 0.1-5 µg/ml fortification level, and 0.1-10 µg/ml at fortification level, respectively, are shown in (*Table 3*). The LOD and LOQ were found to be 1.04 and 3.15 µg/ml for imazapic, and 0.135 and 0.411 µg/ml for imazapyr, respectively, in the top 15 cm. In the extracted soil sample, it was 0.19 µg/ml for imazapic and 0.04 µg/ml for imazapyr. This proves the slow degradation process of these residues in the soils under environmental conditions. The soil samples were taken during rice crop cultivation of about 90 days and the residues are evidently still present.

The Koc for the two herbicides were 137 and 100 ml g^{-1}, respectively, which means low adsorption and high mobility, and eventually high levels of leaching. Nevertheless, both herbicide residues are still present after ~90 days, especially imazapic with 0.2 µg/ml, which was proven by a previous study stating that these types of herbicides are highly persistent (Souza et al., 2016). Simultaneously, persistence of residues in the soil does not necessarily mean that it injures sensitive crops, as persistence differs from bioavailability.

Table 3. *Recovery of imazapic from soil*

Con. (µg/ml)	Recovered peak imazapic	Recovered peak imazapyr	Average % imazapic	Recovery % imazapyr
0.1	2475	3943.66	71.18	107.165
0.5	43513	17263.6	79.19	80.74
5	236358	137529.6	77.27	76.768
10	521185	341438.3	75.75	96.253

Repeatability and stability

The repeatability of this method was determined by calculating the RSD of the peak areas of the six duplicate injections of fortified samples which is < 15. It represents the closeness of the results from similar methods, laboratories, and tools. This is achieved via six concentrations, each replicated trice to a total of eighteen times, encompassing the specified range of the procedure. Accuracy = mean ±SD, for imazapic 75.85 ± 3.4, and for imazapyr, it was 90.232±14.

Conclusions

A simple analytical method based on HPLC-UV was developed and validated to determine the IMI residues in the Clearfield® rice soils. It is necessary to monitor the

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presence of herbicides residues in soils and waters and develop methods for reliable analysis, as important tools of regulatory programs to protect the environment. A gradient of mobile phase A (acetonitrile (100%)) and mobile B (purified water acidified with 10% acetic acid (pH adjusted to 2.8)) yields excellent separation and resolution, in a short analysis time, for the two herbicides (less than 7 min), with retention time for imazapyr and imazapic at ~4.6 and 5.9 min respectively. Excellent linearity in the range of injected standard concentrations with a high degree of precision and accuracy could be achieved. Therefore, the proposed analytical method could be useful for detecting the imidazolinone family in agricultural soil and water in the future. Results of this study suggests the need for an extensive research to determine factors affecting the half-life of these herbicides and their contribution to their persistence. Also, further studies are needed on the laboratory level and plant bioassay to evaluate if these residues can indeed cause injuries to other crops.

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INVESTIGATION OF THE IN VITRO REGENERATION OF SOME MEDICAL AND AROMATIC WILD PLANT SPECIES

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Abstract. *Ferula orientalis* L., *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey., and *Eremurus spectabilis* M.Bieb are among the medically and aromatically important wild plant species. This study was conducted to obtain in vitro shoot regeneration from different plant segments in these three species. For this purpose, different plant segments (rhizome, leaf, or single-node explants) were cultured in Murashige and Skoog medium with the addition of either 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5–2.0 mg/l) or 2,4-D combined with 6-Benzylaminopurine (BAP) (1.0–2.0 mg/l). For the single-node explants of *Ferula orientalis* L., the highest shoot number per explant (3.0 shoots/explant) was obtained in the medium with 0.5 mg/l 2,4-D + 2.0 mg/l BAP added. For *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey., the highest shoot number per explant (2.5 shoots/explant) was obtained in the medium with 1.0 mg/l 2,4-D + 2.0 mg/l BAP added. For *Eremurus spectabilis* M.Bieb., shoot formation was not observed in either of the explant types (leaf or rhizome) in any of the medium combinations. In further studies, the combined applications of 2mg/l or above doses of BAP and lower auxin concentrations may be useful for in vitro shoot regeneration in *Ferula orientalis* L. and *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey.
Keywords: *Ferula orientalis L., Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey., Eremurus spectabilis M. Bieb., micropropogation*

Introduction

Ferula orientalis L. (synonym of *Polycyrtus cachroides* Schlecht.) is a plant with high adaptability and is frequently grown in Iran, Afghanistan, and in the East Anatolian Region of Turkey (Hakkari and Van). It is locally referred to as *heliz* or *kevk* (Kaval, 2011). *Ferula orientalis* L. is from the *Apiaceae* family and is a perennial, herbaceous plant that can grow up to 60–100 cm. In addition to its importance as a food source, it has a therapeutic value and can be used as a fodder plant. Young shoots and leaves of the plant, which have a high nutritional value, are boiled and dried after chopping to rid them of their bitter taste. Then, they are added to dishes and Van herbed cheese (Baytop, 1999; Ozturk et al., 2000). In the summer, the shoots and leaves of the plant are cut and left to dry to feed animals in the winter (Baytop, 1999; Cakılcıoglu et al., 2007). The plant's sap is obtained by boiling the roots and is used to treat stomachaches, hemorrhoids, intestinal parasites, eczema, fungal infections, and toxic animal bites. Oils derived from the plant have antifungal and antibacterial properties (Tuzlacı and Dogan, 2010).

Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey. (synonym of *Scandix macrospermum* Wild. ex Spreng.) is a perennial, herbaceous plant that grows in high altitudes and humid habitats and is locally referred to as *mendi* (Kaval, 2011). It is from the *Apiaceae* family, can grow up to 40–60 cm, and has a thin stem that has a mint-like taste. The plant is used by the locals as a spice and vegetable. In addition to its use in Van herbed cheese and local dishes, it can be pickled, added to soups, and consumed fresh (Ozcelik, 1992). Its use as a food source is more common than *Ferula orientalis* L.

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Eremurus spectabilis M.Bieb. is a perennial, herbaceous plant from the *Eremurus* genus of the *Liliaceae* family. The Iran/Turan region, especially Central Asia, is the center of origin of the species. In Turkey, it is a wild plant that grows on the mountains of the Eastern and Southeastern Anatolia Regions. It is locally known as *ciris*, *wild leek*, and *yellow lily*. Its fresh shoots and leaves are consumed as vegetables. In addition to its use as a food source, it has a therapeutic value and is used as an adhesive in industry. Thanks to its similarity to spinach in terms of cooking style, it can be added to bulgur pilaf or roasted and eaten with eggs, and its leaves and stems can be added to Van herbed cheese (Kaval, 2011). The plant is rich in minerals and is a good source of vitamin C and antioxidants (Kaplan and Unal, 2011). Its sap, which is obtained by crushing its leaves, is used in the treatment of fungal diseases and eczema (Tuzlacı and Doğan, 2010), and its juice, which is obtained by boiling its roots, is alledged to be treat diabetes and liver disorders (Tuzlacı and Doğan, 2010; Pourfarzad et al., 2014). The roots of the plant have been reported to contain high amounts of mucilage, and Tuzlaci (1985) reported that the plant contained 30% gum, which is made into an adhesive for use in industry (Gungor, 2002).

In the future, these medically and aromatically important plant species, which are yet to be cultured, will likely be on the verge of extinction as a result of unconsciously collecting the plants from their natural habitats, their irreplaceable use in the making of Van herbed cheese, arbitrary grazing. Therefore, necessary measures should be taken to protect these species. Methods for plant tissue culturing enable the protection of plant biodiversity (Alkowni and Sawalha, 2012). Moreover, thanks to rapid and reliable micropropagation methods, the production of secondary metabolites in medical plants can be increased(Alkowni et al., 2017). Conventional breeding studies on the species from the *Apiaceae* and *Liliaceae* families are time consuming and require labor (Tawfik and Noga, 2002; Zare et al., 2010), but tissue culture methods can shorten the time spent on breeding.

In vitro regeneration studies have been carried out on many species from the *Apiaceae* (Irwani et al., 2010; Zare et al., 2010; Sharma, 2015) and *Liliaceae* families (Baksha et al., 2005; Skoric et al., 2012; Monemi et al., 2014). It has been reported that the micropropagation of some medical plants, endangered species, wild species, and endemic species was achieved with tissue culture methods (Sahrawat and Chand, 2002; Martin, 2002; Thomas and Jacob, 2004; Paunescu and Holobiuc, 2005). However, these methods should be optimized, and proper protocols must be established for each species. The present study investigated how adding different explant types and different growth hormones to the MS medium (Murashige and Skoog, 1962) medium affected the in vitro propagation of these three medically important wild species. This report is also noteworthy for being the first in vitro regeneration study on these species.

Materials and Methods

Plant materials, sterilization and explant preparation

Different plant parts of *Eremurus spectabilis* M.Bieb., *Ferula orientalis* L., and *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey., which were collected from Kırkgeçit Village of Gurpinar County in Van, Turkey, were the material of the study (*Fig. 1*). For *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey and *Ferula orientalis* L., single-node explants were used, and for *Eremurus spectabilis* M.Bieb, leaf and rhizome explants were used. Rhizomes were rinsed with tap water to remove mud and dirt

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and were kept in tap water overnight. Then, they were rinsed 4–5 times with pure water, placed into a cabinet, and kept in 70% ethyl alcohol for 10 seconds. Next, a few drops of Tween 20 were added, and the samples were shaken for 15 minutes in 15% sodium hypochlorite. The *Eremurus spectabilis* M.Bieb rhizomes were sterilized by shaking the rhizomes with bidistilled water 3–5 times, 6 minutes each time.

The surfaces of the leaf explants and the shoots containing single nodes were sterilized by shaking the samples in 15% commercial sodium hypochlorite for 15 minutes. Then, they were rinsed with bidistilled water 3 times, 5 minutes for each repetition (*Fig. 2a, 2b, 2c, 2d*). Sterilized materials were put on a sterile blotting paper and cleansed of their excess water (*Fig. 2e, 2f*). Rhizomes were prepared for planting by vertically and horizontally cutting their sides in strips (*Fig.2g, 2h, 2l*). To obtain the single-node explants, the nodes that also had shoots half a centimeter above and below were taken and their leaves were removed (*Fig. 2i, 2j*). To obtain leaf explants, thin strips were taken from the sides and edges of the leaves and prepared for planting (*Fig. 2k*). All steps were carried out under aseptic conditions in a laminar flow cabinet.

Figure 1. *(a) Eremurus spectabilis M.Bieb., (b) Ferula orientalis L., (c) Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey plants, (d) Eremurus spectabilis M. Bieb. roots and rhizomes.*

Medium composition and culture of explants

The explants were cultured in the MS (Murashige and Skoog, 1962) media containing 9 different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5, 1.0, and 2.0 mg/l) and/or benzylaminopurine (BAP) (1.0 and 2.0 mg/l). In addition, 30 g/l of

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sucrose and 7 g/l of agar was added to the MS medium, and the pH of the medium was adjusted to 5.8. Each petri dish contained 8 explants and 25 ml of medium. Then, the petri dishes were cultured in a climate room at 25°C ± 2°C with a photoperiodic cycle of 16-hours light/8-hours dark. Each application was planned to be in 4 replicates. The data were evaluated by analyses of variance, and the means were compared by Duncan's multiple range test ($p < 0.05$) using the SPSS statistical package program.

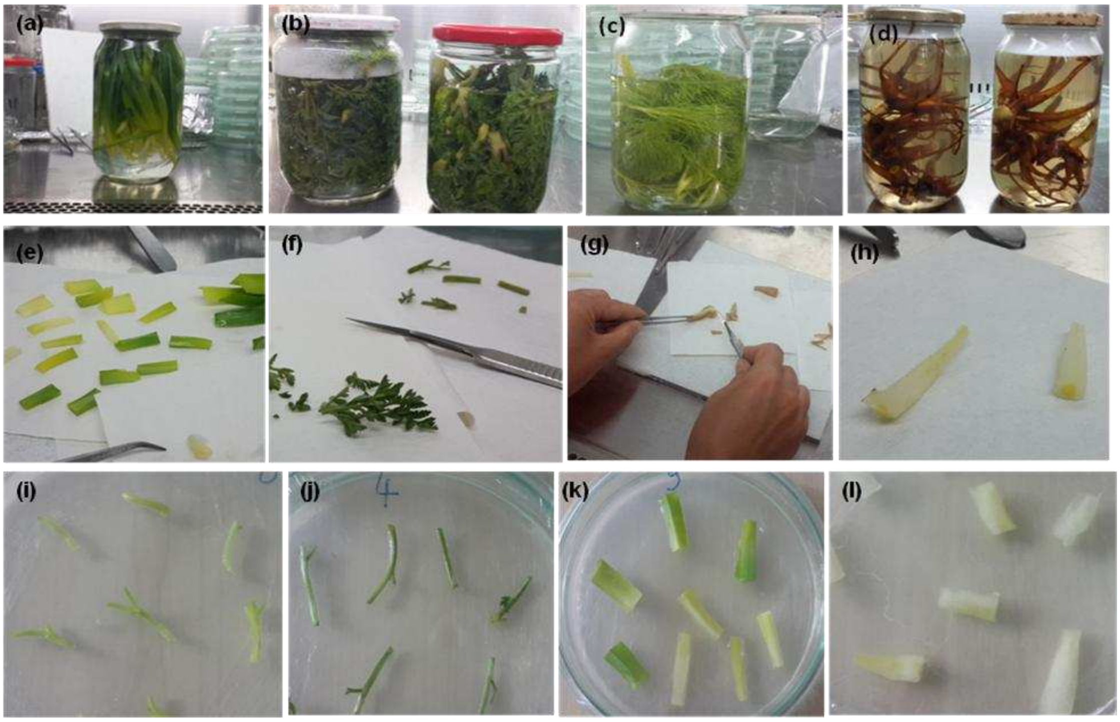

Figure 2. *Surface sterilization of the leaves of (a) Eremurus spectabilis M.Bieb, (b) Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey, and (c) Ferula orientalis L.; (d) sterilization of the rhizomes of Eremurus spectabilis M. Bieb; (e) leaf explants of Eremurus spectabilis M. Bieb; (f) single-node explants of Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey; (g-h) preparation of the rhizome explants of Eremurus spectabilis M. Bieb; (i) single-node explants of Ferula orientalis L.; and (j) single-node explants of Chaerophyllum macrospermum (Spreng.) Fisch. & C.A. Mey, which showed growth in the media 1 week after planting; (k) leaf explants; and (l) rhizome explants of Eremurus spectabilis M. Bieb.*

Results and Discussion

Table 1 shows the media composition with the shoot numbers and ratios (%) obtained from the single-node explants of *Ferula orientalis* L. and *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey. For both species, differences among the medium combinations were statistically significant ($p < 0.05$). For *Ferula orientalis* L., the highest shoot number per explant was obtained in the MS7 medium (3.0 shoot/explant), followed by the MS8 (2.75 shoots/explant) and MS4 (2.25 shoots/explant) media, respectively. For *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A. Mey, the highest shoot number per explant was obtained in the MS8 medium (2.50 shoots/explant), followed by the MS7 medium (2.25 shoots/explant). For both species, the least successful media (in terms of shoot numbers and ratios) were the

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media with only auxin added (2,4-D), which were the MS1 (0.5 mg/l 2,4-D), MS2 (1.0 mg/l 2,4-D), and MS3 (2.0 mg/l 2,4-D) media (*Table 1*).

Fig. 3 shows shoot formations in these wild plant species for different explant types and MS medium combinations. It also shows the browning in the explants that occurred in the successive stages of culture.

Table 1. *Effect of the combinations of MS medium on in vitro shoot regeneration in the single-node explants of Ferula orientalis L. and Chaerophyllum macrospermum (Spreng.) Fisch. & C.A.Mey.*

Medium No	Media composition (mg/l)		*Ferula orientalis*				*Chaerophyllum macrospermum*			
	2,4-D	BAP	The number of cultured explants	The number of shoot-forming explants	Shoot / explant	Shoot ratio (%)	The number of cultured explants	The number of shoot-formng explants	Shoot / explant	Shoot ratio (%)
MS1	0.5	-	32	3	0.75 cd	9.38 cd	32	0	0.00 d	0.00 d
MS2	1.0	-	32	2	0.50 cd	6.25 cd	32	0	0.00 d	0.00 d
MS3	2.0	-	32	0	0.00 d	0.00 d	32	0	0.00 d	0.00 d
MS4	0.5	1.0	32	9	2.25 ab	28.13 ab	32	6	1.50 bc	18.75 bc
MS5	1.0	1.0	32	5	1.25 b-d	15.63 b-d	32	7	1.75 a-c	21.88 a-c
MS6	2.0	1.0	32	7	1.75 a-c	21.88 a-c	32	6	1.50 bc	18.75 bc
MS7	0.5	2.0	32	11	3.00 a	34.38 a	32	9	2.25 ab	28.13 ab
MS8	1.0	2.0	32	10	2.75 a	31.25 ab	32	10	2.50 a	31.25 a
MS9	2.0	2.0	32	5	1.25 b-d	15.63 b-d	32	4	1.00 c	12.50 c
LSD.05					1.16	14.71			0.90	11.34

Different small letters in a same column show significant differences among the medium ($p < 0.05$).

The ratios of the growth regulators from the auxin and cytokinin group in the nutrient medium are important for in vitro plant regeneration (Thomas and Maseena, 2006; Irvani et al., 2010). For both species, *Table 1* reveals that the medium combinations that obtained relatively higher shoot numbers had low levels of auxin (2,4-D) and high levels of BAP. There have been reports on the positive effect of adding combinations of auxin at low concentrations and growth regulators from the cytokinin group to the nutrient medium on in vitro plant regeneration (Baksha et al., 2005; Skoric et al., 2012; Monemi et al., 2014). These reports agree with the present study.

Monemi et al. (2014) reported that for leek (*Allium ampeloprasum*) from the *Liliaceae* family, the best callus formation was obtained in leaf explants in an MS medium with 0.5 mg/l 2,4-D + 0.1 mg/l BAP and 0.1 mg/l 2,4-D + 0.5 mg/l kinetin added. In the present study, callus formation was not observed for any of the three species, even in the media with only 2,4-D added (0.5, 1.0, and 2.0 mg/l). This suggests that using higher doses of auxin to obtain callus formation may prove beneficial in further in vitro regeneration studies on the same species. Zare et al. (2010) reported that, in different explant types (root, hypocotyl, and cotyledon) obtained from sterile

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seedlings of medically important *Ferula assa-foetida* L., indirect shoot formation was obtained both in only BA-added (1–3 mg/l) and in 0.2–0.5 mg/l NAA-added MS medium combinations. Zare et al. (2010) determined that the highest shoot formation (7.4 shoot/callus) was obtained in the hypocotyl explants in a 1 mg/l BA-added and 0.2 mg/l NAA-added medium. In the present study, direct shoot formation from the explants was obtained in two species (*Ferula orientalis* L. and *Chaerophyllum macrospermum* (Spreng). Fisch. & C.A.Mey.). Irvani et al. (2010) reported that the highest shoot regeneration (87.3%) was obtained in the medically important *Dorema ammoniacum* D. (*Apiaceae*) from calluses of hypocotyl origin in a MS + 2 mg/l BA + 0.2 mg/l IBA medium.

Figure 3. *Shoot formation of the single-node explants of Ferula orientalis L. at the (a) 15th day of culturing and at the (b, c) 25th day of culturing (0.5 mg/l 2,4-D + 2.0 mg/l BAP), shoot formation of the single-node explants of Chaerophyllum macrospermum (Spreng.) Fisch. & C.A. Mey at the (d) 25th day of culturing (1.0 mg/l 2,4-D + 2.0 mg/l BAP) and at the (e) 15th day of culturing (1.0 mg/l 2,4-D + 1.0 mg/l BAP), (f) swellings in leaf explants of Eremurus spectabilis M. Bieb at the 15th day of culturing (0.5 mg/l 2,4-D + 1.0 mg/l BAP), and (g-i) browning in the explants.*

The medium combinations affected swelling, browning, and infection development in the explants (*Tables 2 and 3*). Growth and enlargement in the explants in the nutrient media were accepted as swelling. For *Ferula orientalis* L., the highest swelling ratio

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was observed in the MS7 (100%) and MS8 (93.8%) media. For *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey., maximum swelling was also observed in the same medium combinations (*Table 2*). For *Ferula orientalis* L., the browning ratio in the cultured single-node explants varied between 46.9% and 78.1%, and the infection ratio in the explants varied between 15.6% and 28.1%. For *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey., the same parameters varied between 53.1% and 84.4% and between 12.5% and 25.0%, respectively. For both species, shoot formation increased with increasing swelling ratios (*Table 2*). *Table 3* is given the effects of explant type and medium combination on swelling, browning, and infection development in *Eremurus spectabilis*. In rhizome explants, no swelling was observed in any of the medium combinations, whereas, in leaf explants, 100% swelling was observed in all medium combinations. However, shoot formation was not obtained in any of the medium combinations for either of the explant types (*Table 3*).

Table 2. *Effect of medium combination on the swelling, browning, and infection of the single-node explants of Ferula orientalis L. and Chaerophyllum macrospermum (Spreng.) Fisch. & C.A. Mey.*

Medium No	Media composition (mg/l)		*Ferula orientalis*						*Chaerophyllum macrospermum*					
	2, 4-D	BAP	The number of swelling explant	Swelling explant ratio (%)	The number of browning explant	Browning explant ratio (%)	The number of infection developing explant	Infection ratio (%)	The number of swelling explant	Swelling explant ratio (%)	The number of browning explant	Browning explant ratio (%)	The number of infection-developing explant	Infection ratio (%)
MS1	0.5	-	12	37.5	20	62.5	9	28.1	13	40.6	27	84.4	5	15.6
MS2	1.0	-	19	59.4	25	78.1	5	15.6	10	31.3	26	81.3	6	18.8
MS3	2.0	-	20	62.5	25	78.1	7	21.9	12	37.5	24	75.0	8	25.0
MS4	0.5	1.0	27	84.4	18	56.3	5	15.6	16	50.0	20	62.5	6	18.8
MS5	1.0	1.0	20	62.5	18	56.3	9	28.1	16	50.0	18	56.3	7	21.9
MS6	2.0	1.0	22	68.7	19	59.4	6	18.8	18	56.3	21	65.6	5	15.6
MS7	0.5	2.0	32	100	16	50.0	5	15.6	27	84.4	19	59.4	4	12.5
MS8	1.0	2.0	30	93.8	15	46.9	7	21.9	30	93.8	17	53.1	5	15.6
MS9	2.0	2.0	21	65.6	18	56.3	9	28.1	14	43.8	20	62.5	8	25.0

In rhizome explants, browning ratios and infection ratios varied between 50% and 79.2% and between 20.8% and 50%, respectively. In leaf explants, the values varied between 68.8% and 100% and between 6.3% and 31.5%, respectively (*Table 3*). Browning that occurs under in vitro conditions causes losses in the vitality of tissues in many phenolic compound–rich species. It is known that adding phenol-captivating substances such as polyvinylpyrrolidone (PVP), active carbon (AC), and silver nitrate ($AgNO_3$) to nutrient media can prevent tissue browning by captivating polyphenol compounds and thereby slowing their synthesis (Teixeira et al., 2006; Alp et al., 2010). Therefore, we presume that in further studies on the same species, adding these compounds to nutrient media may help reduce losses due to browning. Moreover, there

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are reports on the phytotoxic effects of antibiotics, which are added to nutrient media to prevent infections, and their browning-increasing effect on tissues. In the present study, infection development in explants were observed at varying ratios depending on the species and media composition In further studies on these species, using explants from sterile seedlings that are developed under in vitro conditions will minimize infections.

Table 3. *Effect of medium combination and explant types on swelling, browning, and infection development of the rhizome and leaf explants of Eremurus spectabilis M. Bieb.*

Medium No	Media compositon (mg/l)		The number of swelling explant		Swelling explant ratio (%)		The number of browning explant		Browning explant ratio (%)		The number of infection-developing explant		Infection ratio (%)	
	2,4 -D	BAP	Rhizme	Leaf	Rhizme	Leaf	Rhizme	Leaf	Rhizme	Leaf	Rhizme	Leaf	Rhizme	Leaf
MS1	0.5	-	-	16	-	100	17	16	70.8	100	7	-	29.2	-
MS2	1.0	-	-	16	-	100	19	15	79.2	93.8	5	1	20.8	6.3
MS3	2.0	-	-	16	-	100	17	14	70.8	87.5	7	2	29.2	12.5
MS4	0.5	1.0	-	16	-	100	18	15	75.0	93.8	6	1	25.0	6.3
MS5	1.0	1.0	-	16	-	100	15	13	62.5	81.3	9	3	37.5	18.9
MS6	2.0	1.0	-	16	-	100	14	11	58.3	68.8	10	5	41.7	31.5
MS7	0.5	2.0	-	16	-	100	12	11	50.0	68.8	12	5	50.0	31.5
MS8	1.0	2.0	-	16	-	100	12	13	50.0	81.3	12	3	50.0	18.9
MS9	2.0	2.0	-	16	-	100	13	12	54.2	75.0	11	4	45.8	25.0

Conclusions

This study is the first report of the effects of different explant types and medium combinations on the in vitro regeneration of the medically and aromatically important *Ferula orientalis* L., *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey. and *Eremurus spectabilis* M.Bieb. species. Shoot formations were obtained in the single-node explants of *Ferula orientalis* L. and *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey. at ratios that varied depending on the medium composition. However, shoot formation was not observed for *Eremurus spectabilis* M.Bieb. in either of the explant types in any of the medium combinations. In further studies, trying different hormones from the auxin-cytokinin group in addition to the combined applications of 2mg/l or above doses of BAP and lower auxin (0.1, 0.2, and 0.5 mg/l) concentrations may prove beneficial for in vitro shoot regeneration in *Ferula orientalis* L. and *Chaerophyllum macrospermum* (Spreng.) Fisch. & C.A.Mey. The results of the present study are expected to contribute to in vitro regeneration studies on these medicinal plants in future.

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POPULATION GENETIC STRUCTURE OF *ORTHOTOMICUS EROSUS* (WOLLASTON, 1857) (COLEOPTERA: CURCULIONIDAE, SCOLYTINAE) IN PINE FORESTS OF THE MEDITERRANEAN REGION OF TURKEY

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Abstract. *Orthotomicus erosus* (Wollaston, 1857) (Coleoptera: Curculionidae: Scolytinae) is one of the principal pests of Eurasian forests. Gathering data from this study concerning population genetic structure of *O. erosus* would help improving new strategies to control the pest by giving new insights on forest management. The aims of the study were to resolve the population genetic structure of *Orthotomicus erosus* which distributes Mediterranean Region of Turkey and to determine the factors (such as host pine species and geographic barriers) that contributed to the current distribution of genetic diversity. The beetle samples were collected from 20 stands from the pine forests included in the Mediterranean Region of Turkey. 67 samples were studied using mitochondrial cytochrome oxidase subunit I (COI) gene and Neighbor Joining (NJ) and Maximum Likelihood (ML) analyses were performed. As a result of the study, thirty-seven distinct haplotypes from sixty-seven samples were determined. The species did not form any phylogroup in populations depending on the geographical location according to the NJ and ML analysis. The NJ and ML trees revealed that those *O. erosus* individuals that fed on different pine species did not have genetic variations. Consequently, NJ and ML analysis results reveals that different populations of the species across the Mediterranean Region were not disconnected and isolated geographically either.
Keywords: *Orthotomicus erosus, mtDNA, Neighbour Joining, Maximum Likelihood, Turkey*

Introduction

Forests have played a very important role for all organisms since the existence of human-beings. However, forestlands have shrunk and have been destroyed due to overutilization and misuse for centuries. Particularly the growing world population in the last century and thus the social pressure as well as the negative impacts such as environmental pollution, forest fire and insect damage have resulted in the shrinkage of forest areas, which still prevails at the same pace (Sarıkaya and Avcı, 2006).

There are several factors that led to shrinkage of forest areas and reduction of their productivity in the Mediterranean Region located in the south of Turkey. Among these factors, harmful insect species play a very important role. Bark beetles are among the most important insect groups that lead to significant economic loss due to the damage they cause in forest trees. The bark beetles that damage the forests in the Mediterranean Region include Mediterranean pine engraver *Orthotomicus erosus* (Wollaston, 1857), which is highly important because of its damage in pine forests. Although these beetles cause secondary damage, they may cause primary damage just after the dry periods. In this region, especially *Pinus brutia* is one of most widely distributed species (Karatepe et al., 2014). *Orthotomicus erosus* (Wollaston) was found in pine (*Pinus* spp.), fir (*Abies*

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spp.), spruce (*Picea orientalis*) and cedar (*Cedrus libani*) species in Turkey (Schedl, 1961; Tosun, 1975; Selmi, 1989 and 1998; Çanakçıoğlu and Mol, 1998). Studies conducted in other countries demonstrated that *O. erosus* caused damage in pine species in primarily Mediterranean countries and Middle and South Europe, Iran, Israel, Morocco, Tunisia, Algeria, Crimea, Caucasia, South and North America (Mendel and Halperin, 1982; Mendel, 1983; Pfeffer, 1995; Henin and Pavia, 2004; Lee, 2004; Haack, 2004; Jamaa et al., 2007; Amini et al., 2013; Gómez and Martínez, 2013).

Molecular tests have been increasingly used in systematic and taxonomic studies conducted on insects and contributed to the solution of taxonomic problems thanks to the recent developments, enzymatic amplification of specific regions of a DNA strand In Vitro (PCR), (Mullis et al., 1986), introduction of automatic devices for DNA sequence analysis and superfast computers (Brower and De Salle, 1994; Roderick, 1996; Simon et al., 1994). Current population structure of a species reflects both historic and contemporary ecological and evolutionary forces (Hewitt, 2000). These processes left their signatures in the genomes of species. Thus, current population genetic structure can be used to infer past evolutionary and demographic events within species (Avise, 2000).

There is any study concerning population genetics of *O. erosus* (Hughes and Vogler 2004) although the species has been included in several molecular studies (Cognato and Felix, 2000; Cognato and Vogler, 2001; Jordal et al., 2008; Cognato, 2013; Jordal and Kambestad, 2014). *O. erosus* is one of the most damaging bark beetle species to pine forests at the Mediterranean shore latitude in Turkey. Generally a secondary pest, it can immediately gain a primary character especially after the arid periods in the Mediterranean and Aegean regions of Turkey. However, population structure of the species is also unknown in Turkey. Information concerning its population structure could help in the future control of this pest. The aims of the study were to resolve the population genetic structure of *O. erosus* in the Mediterranean Region of Turkey and to determine the factors (such as host pine species and geographic barriers) that contributed to the current distribution of genetic diversity.

Material and Methods

In order to collect *Orthotomicus erosus* samples, sampling plots were selected in the stands where the Brutian pine and black pine grew under different habitat conditions in the Mediterranean Region and the damage caused by the bark beetle was intense and beetle samples were collected from these areas. The adult samples of *Orthotomicus erosus* were collected from different locations across the Mediterranean Region in 2013. The locations where *O. erosus* samples were collected are presented in *Table 1* and *Figure 1*.

Table 1. *Pinus brutia and P. nigra stands where Orthotomicus erosus samples were collected. (Host tree species were symbolised with capital letter N: P. nigra; B: P. brutia in the Abbreviation column)*

Samples No	Abbreviation	Location	Coordinates	Altitude (m)	Host species
1	B-AntKem	Kemer-Karabucak	N 36° 58′ 31′′ E 30° 53′ 45′′	44	*Pinus brutia*
2	B-AntAks	Akseki-Küser	N 37° 10′ 86′′ E 31° 74′ 35′′	1127	*P. brutia*
3	B-AntKum	Antalya-Kumluca	N 36° 23′ 56′′ E 30° 19′ 22′′	300	*P. brutia*

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4	N-AntAksCam	Akseki-Çambeleni	N 37° 14′ 87′′ E 31° 83′ 85′′	1367	*P. nigra*
5	N-BurAkc	Burdur-Akçaören	N 37° 41′ 64′′ E 30° 19′ 91′′	1305	*P. nigra*
6	B-BurBuc	Burdur-Bucak Gündoğdu	N 37° 35′ 10′′ E 30° 61′ 66′′	892	*P. brutia*
7	B-IspEgiAsa	Eğirdir-Aşağıgökdere	N 37° 32′ 88′′ E 30° 48′ 45′′	250	*P. brutia*
8	B-MrsTarCam	Tarsus Çamalanı Akarca	N 37° 31′ 28′′ E 34° 78′ 45′′	1200	*P. brutia*
9	B-MerGl	Mersin-Gülnar	N 36° 32′ 97′′ E 33° 40′ 40′′	956	*P. brutia*
10	B-MerAna	Anamur-Demirören village	N 36° 06′ 57′′ E 32° 65′ 62′′	254	*P. brutia*
11	B-MerTar	Mersin-Tarsus	N 37° 13′ 04′′ E 34° 54′ 36′′	950	*P. brutia*
12	B-MrsDav	Mersin-Davultepe	N 36° 48′ 49′′ E 34° 22′ 14′′	760	*P. brutia*
13	N-MerGz	Mersin-Gözne	N 37° 04′ 30′′ E 34° 33′ 30′′	1200	*P. nigra*
14	N-MrsTarCam	Tarsus- Çamalanı, Tekir	N 37° 31′ 28′′ E 34° 78′ 45′′	1325	*P. nigra*
15	B-AdaCuk	Adana-Çukurova Karahan Köyü-Tapan Tepe	N 37° 08′ 18′′ E 35° 17′ 82′′	141	*P. brutia*
16	B-AdaKad	Osmaniye-Kadirli-Börklüler	N 37° 47′ 17′′ E 36° 20′ 26′′	627	*P. brutia*
17	B-OsmYar	Osmaniye-merkez Yarpuz-Yemşen	N 37° 07′ 21′′ E 36° 33′ 94′′	728	*P. brutia*
18	N-AdaKar	Karaisalı-Damlama	N 37° 35′ 76′′ E 34° 96′ 76′′	1271	*P. nigra*
19	B-KmrAnd	Andırın-Efirağızlı	N 37° 51′ 78′′ E 36° 37′ 74′′	625	*P. brutia*
20	N-KmrAndSar	Andırın-Sarıtanışmanlı	N 37° 67′ 44′′ E 36° 45′ 80′′	1436	*P. nigra*

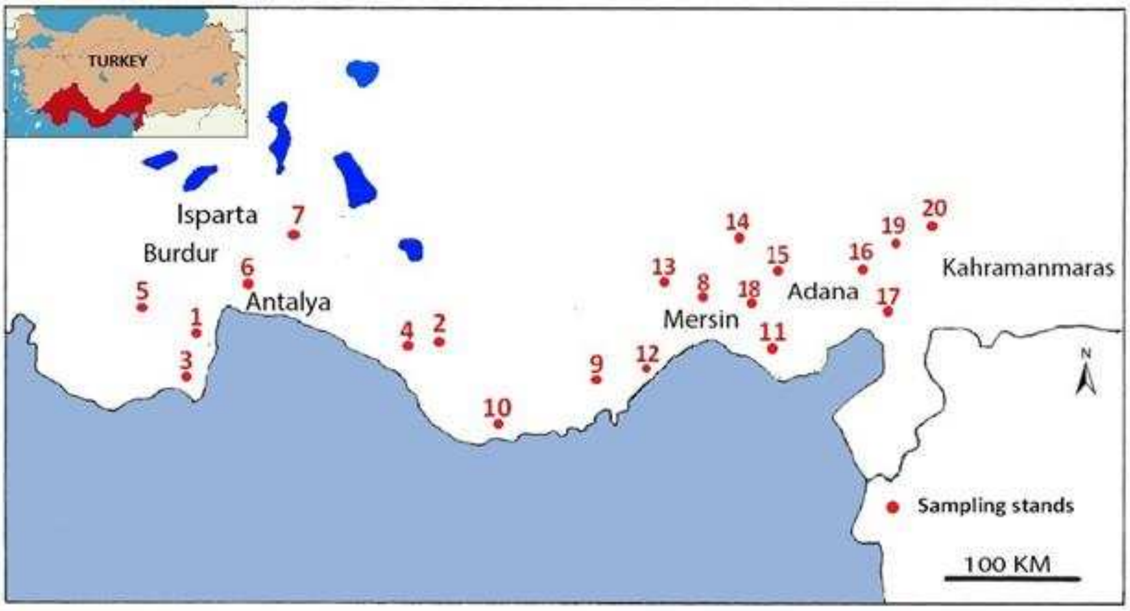

***Figure 1.** Sampling stands of Orthotomicus erosus specimens*

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Trap woods were placed in the sampling plots before the flying period in order to collect the samples. Traps were composed of 12 woods each with a length of 1 m. The samples collected from the trap trees were soaked in pure alcohol of 99% and stored.

Furthermore, pheromone traps were placed in the sampling plots before flying periods and the samples were collected from these traps. Pheromone preparations containing 1500 mg methyl butanol + 100 mg cis-verbanol + 30 mg Ipsdienol were used in the pheromone traps to capture *O. erosus*. Moreover, samples were collected from trunks maintained in the forest storages and production areas across the region through observation. The collected samples were placed and stored in tubes containing pure alcohol. The tubes where the samples were placed were stored in deep freezer until the performance of genomic DNA (gDNA) isolation.

Genomic DNA isolation

gDNAs of *O. erosus* samples collected during the field study were isolated from the head and thorax of the beetles. Abdominal and elytra of the samples from which gDNA was isolated were stored at the laboratory for morphological analysis. gDNAs of the samples were isolated with Qiagen DNeasy Blood and Tissue Kit (Qiagen).

Polymerase Chain Reaction (PCR) and DNA sequencing

The isolated gDNAs were controlled using 0.8% agarose gel electrophoresis and the gDNA quantity of the samples was diluted to the concentration level that was appropriate for PCR according to their gel appearance. The primers S1718 (5'-GGAGGATTTGGAAATTGATTAGTTCC-3') and A2237 (5'-CCGAATGCTTCTTTTTTACCT CTTTCTTG-3') were used for the amplification of mitochondrial cytochrome oxidase subunit I (COI) regions of the samples through PCR (Simon et al., 1994; Normark et al. 1999). Thirty cycles of amplification were performed as follows: denaturation step at 94°C for 30 seconds, annealing at 48°C for 1 min, and extension at 72°C for 90 seconds. PCR products were purified with QIAquick PCR purification kit (QIAgen) and sequenced.

Population Genetic Analyses

The phylogenetic relationships between the haplotypes were analysed and phylogenetic trees were created using Neighbour Joining (NJ) and Maximum Likelihood (ML) algorithms. The best substation model according to the DNA sequencing data was determined using the hierarchical likelihood ratio test (hLRT) and Akaike information criterion (AIC) in MODELTEST v. 3.7 (Posada and Crandall, 1998). The substitution model for the two abovementioned statistics was found to be TIM3+I for upon *Orthotomicus erosus* by consensus and these criteria were entered as substitution model while constructing the Maximum Likelihood (ML) tree. ML trees were questioned according to the heuristic search approach. The branch support values of ML trees were evaluated through 1000 non-parametric bootstrap analysis (Felsenstein, 1985).

In order to determine the polymorphism and diversity level of *Orthotomicus erosus*, the number of within populations and among populations average nucleotide differences and nucleotide diversity (π) were analysed. In order to determine the significance of the differences between the populations of the species, pairwise Fst values were calculated.

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In order to determine if the populations of the species showed the cases such as enlargement/bottleneck in the past, historical demographic pattern of both species were analysed. To this end, Tajima's D (Tajima, 1989) and Fu's FS (Fu, 1997) neutrality tests were performed for the COI gene regions that were studied. Moreover, sum of squared differences (SSD) and Harpending's Raggedness index (Hri) mismatch distribution analysis were used to check if the sequence data of the species deviated from the possible enlargement model estimation.

Results and Discussion

67 samples were analysed for *Orthotomicus erosus* in this study. The DNA sequences of the samples that were studied were aligned, the impractical regions were removed, and a sequence of 488 base pairs that could be used for statistical analysis was obtained. 49 bases out of the sequence of 488 base pairs had mutations. 37 different haplotypes were found in 67 samples that were analysed due to the abovementioned mutations. Some haplotypes were represented more than once.

The within populations and among populations genetic variations of *Orthotomicus erosus* were analysed through AMOVA (*Table 2*). The analysis showed that the within populations genetic variation of the species was higher than the among populations variation.

Table 2. *Spatial analysis of molecular variance of Orthotomicus erosus. df degrees of freedom*

	d.f.	Sum of squares	Variance components	Percentage of variance
among populations	18	242.876	3.11977 Va	55.07
within populations	48	122.183	2.54549 Vb	44.93
Total	66	365.060	5.66525	

The genetic diversity of 67 samples from different populations of *Orthotomicus erosus* collected from the Mediterranean Region and intraspecific genetic diversity were determined with Neighbour Joining (*Figure 2*) and Maximum Likelihood (*Figure 3*) methods. According to the base data obtained prior to the Maximum Likelihood analysis, the substitution model in the COI sequence of the species was found to be TIM3+I in Model test software and the tree was constructed according to that substitution model. To construct both trees, *O. erosus*, *O. proximus* and *O. suturalis* were used as the outgroup.

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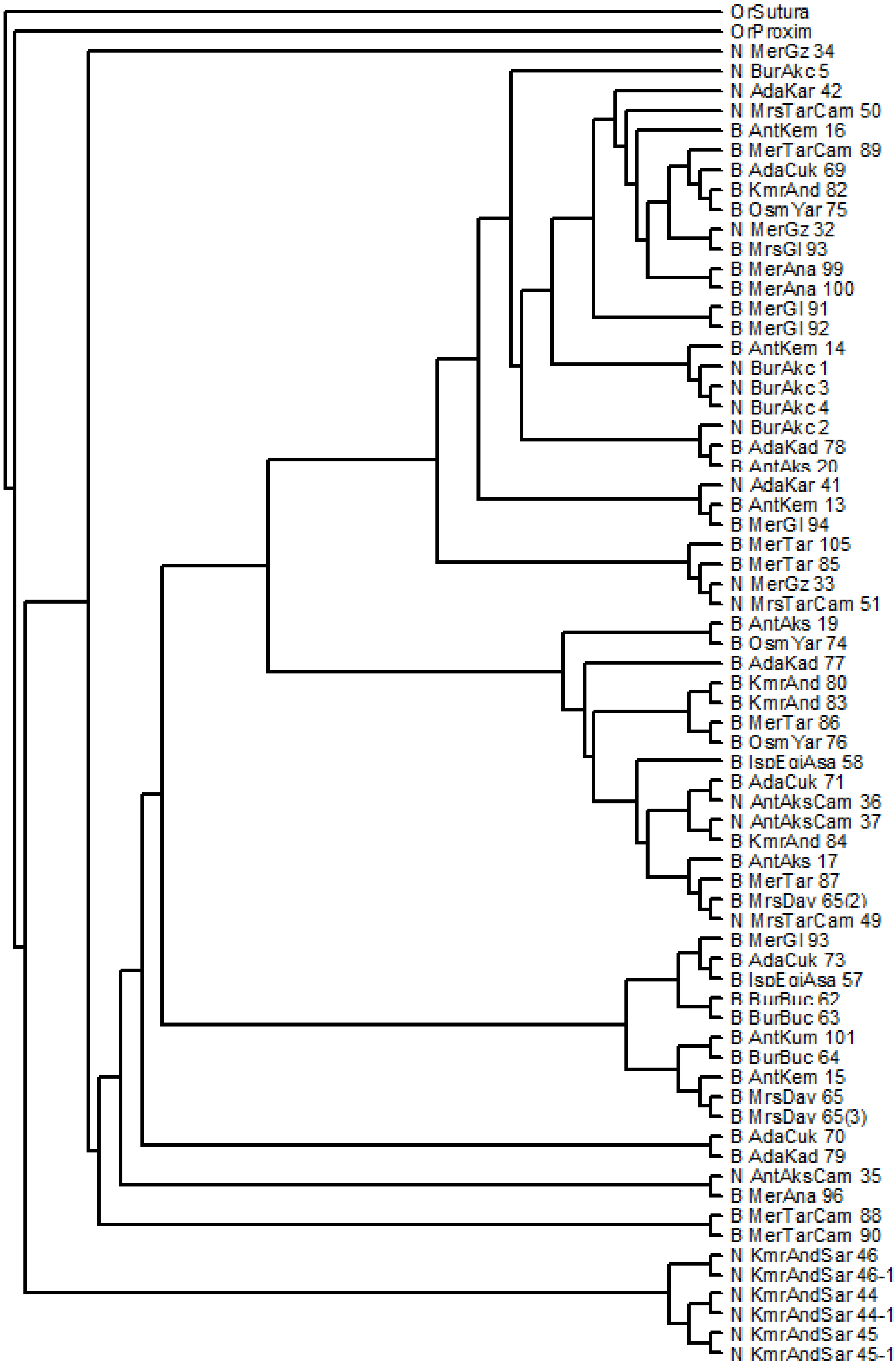

Figure 2. *Neighbour Joining tree of O. erosus populations*

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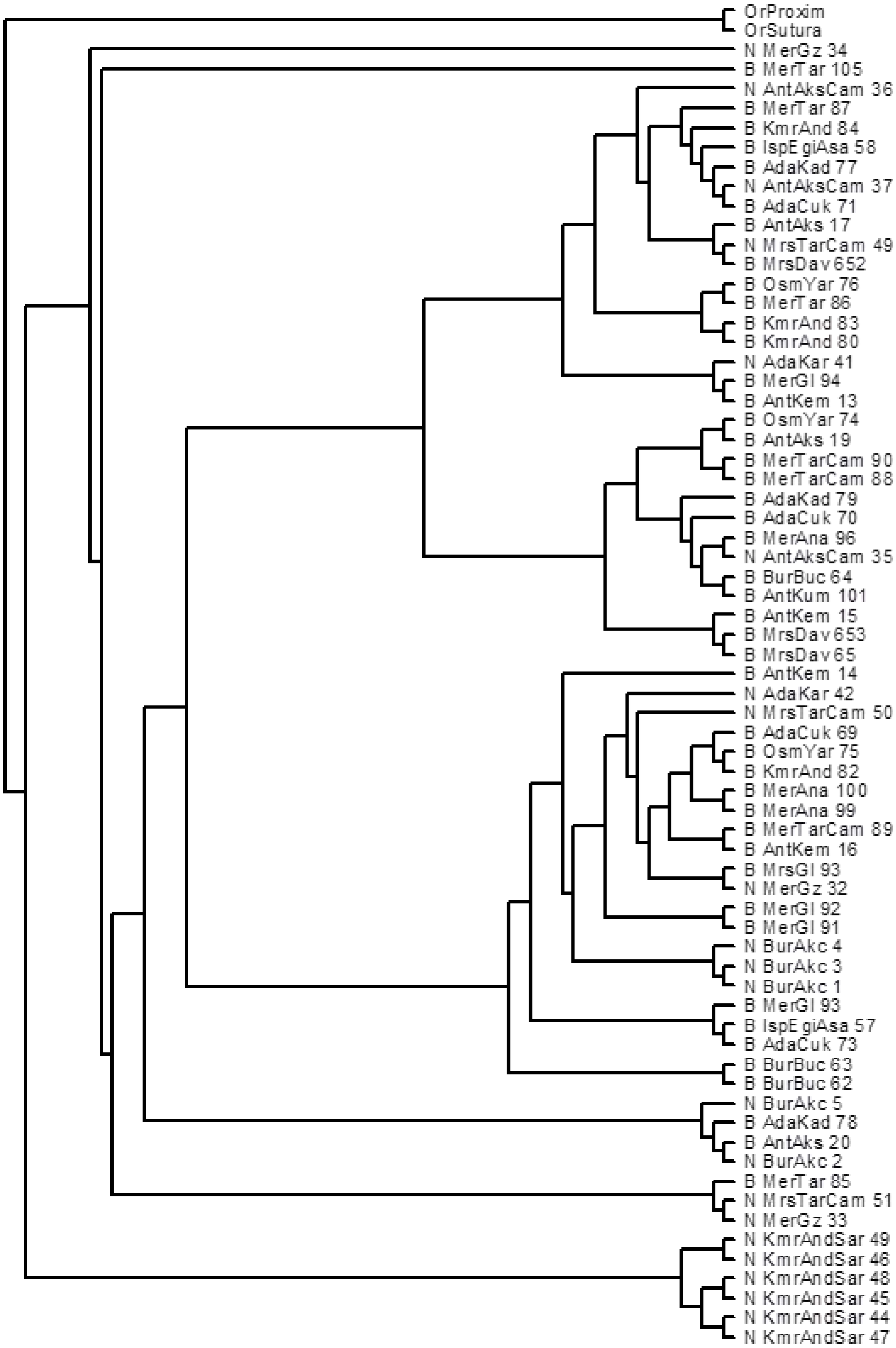

Figure 3. *Maximum Likelihood tree of O. erosus populations*

The intra-population genetic diversity of different populations of *Orthotomicus erosus* in the Mediterranean Region and the results of the neutrality test are presented in *Table 3*. The mismatch distribution analysis of different populations of *Orthotomicus erosus* in the Mediterranean Region is shown in *Table 4*; and the FST values of those populations are shown in *Table 5*.

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Table 3. *Genetic diversity of Orthotomicus erosus populations in the Mediterranean Region and the results of the neutrality test; h: haplotype diversity, π: nucleotide diversity*

	B_AdaCuk	B_AdaKad	N_AdaKar	B_AntAks	N_AntAksCam	B_AntKem_Kum	N_BurAkc	B_BurBuc	B_IspEgiAsa	B_KmrAnd	N_KmrAndSar
Individual number	4	3	2	3	3	5	5	3	**2**	**4**	**6**
3h3	1.0000 +/- 0.1768	1.0000 +/- 0.2722	1.0000 +/- 0.5000	1.0000 +/- 0.2722	1.0000 +/- 0.2722	1.0000 +/- 0.1265	1.0000 +/- 0.1265	1.0000 +/- 0.2722	1.0000 +/- 0.5000	1.0000 +/- 0.1768	1.0000 +/- 0.0962
π	0.016052 +/- 0.011315	0.013661 +/- 0.011061	0.016393 +/- 0.017388	0.010929 +/- 0.009016	0.002732 +/- 0.002807	0.013525 +/- 0.008996	0.002049 +/- 0.001908	0.008197 +/- 0.006966	0.010246 +/- 0.011224	0.012295 +/- 0.008853	0.005464 +/- 0.003896
Theta (*S*)	8.18182	6.66667	8.00000	5.33333	1.33333	7.20000	0.96000	4.00000	5.00000	6.54545	2.18978
Theta (*S*) s.d.	4.74042	4.32784	6.0000	3.52767	1.09834	3.92508	0.75803	2.72554	3.87298	3.85876	1.34160
Theta (*π*)	7.83333	6.66667	8.00000	5.33333	1.33333	6.60000	1.00000	4.00000	5.00000	6.00000	2.66667
Theta (*π*) s.d.	5.52152	5.39776	8.48528	4.39978	1.36987	4.38999	0.93095	3.39935	5.47723	4.32049	1.90127
Tajima's D	-0.43306	0.00000	0.00000	0.00000	0.00000	-0.60926	0.24314	0.00000	0.00000	-0.84046	121.883
Tajima's D p-value	0.48700	0.70900	100.000	0.75600	0.94000	0.36800	0.76000	0.77100	100.000	0.09800	0.89200
FS	0.04321	0.70320	207.944	0.45758	-121.640	-0.91837	-477.912	0.13353	160.944	-0.28768	-357.660
FS p-value	0.28900	0.44400	0.53600	0.38100	0.06600	0.15800	0.00000	0.28300	0.50100	0.23600	0.00400
	B_MerAna	N_MerGz	B_MerGl	B_MerTar	B_MerTarCam	B_MrsDav	N_MerTarTek	B_OsmYar	Mean	s.d.	
Individual number	**3**	3	5	4	3	3	3	3			
h	1.0000 +/- 0.2722	1.0000 +/- 0.2722	1.0000 +/- 0.1265	1.0000 +/- 0.1768	1.0000 +/- 0.2722	1.0000 +/- 0.2722	1.0000 +/- 0.2722	1.0000 +/- 0.2722			
π	0.016393 +/- 0.013103	0.012295 +/- 0.010039	0.010656 +/- 0.007249	0.005464 +/- 0.004352	0.015027 +/- 0.012082	0.009563 +/- 0.007992	0.016393 +/- 0.013103	0.015027 +/- 0.012082			

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Theta (*S*)	8.00000	6.00000	5.76000	2.72727	7.33333	4.66667	8.00000	7.33333	5.53847	2.33498	
Theta (*S*) s.d.	5.12696	3.92792	3.20568	1.78962	4.72749	3.12694	5.12696	4.72749	3.57554	1.47199	
Theta (π)	8.00000	6.00000	5.20000	2.66667	7.33333	4.66667	8.00000	7.33333	5.45439	2.24756	
Theta (π) s.d.	6.39444	4.89898	3.53761	2.12374	5.89622	3.89998	6.39444	5.89622	4.45448	1.93464	
Tajima's D	0.00000	0.00000	-0.70314	-0.21249	0.00000	0.00000	0.00000	0.00000	-0.07034	0.42527	
Tajima's D p-value	0.70000	0.72500	0.32700	0.59100	0.71100	0.76200	0.70200	0.69600	0.68395	0.23034	
FS	0.90079	0.58779	-128.257	-141.422	0.80681	0.30830	0.90079	0.80681	-0.21775	169.616	
FS p-value	0.44400	0.39300	0.10600	0.06300	0.44200	0.37000	0.44600	0.39600	0.29253	0.17609	

Table 4. *Mismatch distribution analysis of Orthotomicus erosus*

	B_AdaCuk	B_AdaKad	N_AdaKar	B_AntAks	N_AntAksCam	B_AntKem_Kum	N_BurAkc	B_BurBuc	B_IspEgiAsa	B_KmrAnd	N_KmrAndSar
SSD	0.15942	0.20122	0.00000	0.22311	0.26508	0.04733	0.00764	0.36418	0.00000	0.17790	0.09176
SSD p-value	0.26000	0.26000	0.00000	0.45000	0.14000	0.74000	0.83000	0.13000	0.00000	0.15000	0.15000
Hri	0.16667	0.66667	0.00000	0.66667	100.000	0.12000	0.11000	111.111	0.00000	0.33333	0.32444
Hri p-value	0.73000	0.54000	0.00000	0.56000	0.54000	0.68000	0.88000	0.27000	0.00000	0.64000	0.17000

	B_MerAna	N_MerGz	B_MerGl	B_MerTar	B_MerTarCam	B_MrsDav	N_MerTarTek	B_OsmYar	Mean	s.d.
SSD	0.44599	0.36835	0.07231	0.29711	0.44161	0.13406	0.14034	0.21895	0.19244	0.14260
SSD p-value	0.02000	0.10000	0.39000	0.02000	0.01000	0.46000	0.41000	0.27000	0.25211	0.24310
Hri	100.000	111.111	0.20000	111.111	100.000	0.44444	0.22222	0.66667	0.53971	0.41232
Hri p-value	0.49000	0.36000	0.34000	0.02000	0.54000	0.83000	0.91000	0.60000	0.47895	0.28614

Table 5. *Pairwise Fst values of Orthotomicus erosus populations*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0,000																		
2	-0,056	0,000																	
3	-0,214	-0,136	0,000																
4	-0,072	-0,080	-0,063	0,000															
5	-0,031	0,000	0,172	0,000	0,000														
6	0,074	0,128	-0,007	0,089	0,184	0,000													
7	0,138	0,271	0,286	0,152	0,544	0,228	0,000												
8	0,114	0,186	0,213	0,208	0,273	0,161	0,545	0,000											
9	-0,094	-0,031	-0,083	-0,076	0,138	0,115	0,441	0,204	0,000										
10	-0,085	0,031	-0,072	-0,068	0,061	0,134	0,211	0,250	0,016	0,000									
11	0,875	0,895	0,901	0,903	0,936	0,878	0,944	0,917	0,916	0,895	0,000								
12	0,129	0,305	0,040	0,221	0,425	0,249	0,483	0,400	0,222	0,190	0,880	0,000							
13	-0,038	0,123	-0,017	0,105	0,233	0,093	0,326	0,297	0,136	0,122	0,892	0,100	0,000						
14	0,001	0,155	-0,137	0,073	0,249	0,127	0,099	0,305	0,129	0,115	0,894	0,166	0,035	0,000					
15	0,000	0,045	0,158	-0,009	0,045	0,177	0,348	0,373	0,141	0,037	0,924	0,366	0,077	0,210	0,000				
16	0,059	0,171	0,010	0,136	0,304	0,198	0,357	0,320	0,210	0,193	0,884	0,127	0,032	0,149	0,295	0,000			
17	0,093	0,105	0,152	0,118	0,182	0,213	0,503	0,250	0,128	0,168	0,910	0,406	0,200	0,281	0,181	0,325	0,000		
18	-0,066	0,000	-0,091	0,016	0,067	0,095	0,348	0,182	-0,050	0,014	0,884	0,143	-0,125	0,100	0,020	0,138	0,081	0,000	
19	-0,185	-0,068	-0,267	-0,213	0,000	0,032	0,160	0,177	-0,099	-0,167	0,890	0,068	0,000	-0,041	-0,063	0,015	0,069	-0,078	0,000

1	B-AdaCuk	6	B-AntKem-Kum	11	N-KmrAndSar	16	B-MerTarCam
2	B-AdaKad	7	N-BurAkc	12	B-MerAna	17	B-MrsDav
3	N-AdaKar	8	B-BurBuc	13	N-MerGz	18	N-MerTarTek
4	B-AntAks	9	B-IspEgiAsa	14	B-MerGl	19	B-OsmYar
5	N-AntAksCam	10	B-KmrAnd	15	B-MerTar	20	

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The intraspecific diversity of *Orthotomicus erosus* was determined by using COI gene sequence that had 488 base pairs. The species did not form any phylogroup in populations depending on the geographical location according to the NJ and ML analysis. The genetic variations of *O. erosus* populations in *Pinus brutia* and *P. nigra* were also analysed. The NJ and ML trees revealed that those *O. erosus* individuals that fed on different pine species did not have genetic variations. Moreover, NJ and ML trees also showed that different populations of the species across the Mediterranean Region were not disconnected and isolated geographically either.

The number of the haplotypes shared by the populations of *O. erosus* was low. AMOVA results also revealed that the variations within and among populations were very similar. This indicates that the intraspecific genetic diversity was very high.

High genetic diversity of genetic lineages were also reported for other animal species from Turkey (Gündüz et al., 2005; Stone et al., 2007; Bardakçı et al., 2006, Mutun, 2011). High haplotype diversity might be due to the continuous introduction of individuals from different locations.

The highest nucleotide diversity was found in the populations distributed in *Pinus brutia* trees in Adana-Çukurova Karahan Village, Mersin-Anamur-Demirören Village and Mersin-Tarsus and in *P. nigra* trees in Adana Karaisalı-Damlama district, Mersin-Tarsus-Çamalanı, Tekir and Mersin-Gözne.

According to the FST values of *O. erosus*; there was a gene flow between the populations in Adana-Çukurova Karahan village and Adana-Kadirli Börklüler, Antalya-Akseki-Küser district, Akseki-Çambeleni, Adana Karaisalı-Damlama; Isparta, Eğirdir-Aşağıgökdere, Kahramanmaraş Andırın-Efirağızlı, Mersin-Gözne, Mersin-Tarsus-Çamalanı- Tekir and Osmaniye- Yarpuz-Yemşen; populations in Adana-Kadirli Börklüler and Adana Karaisalı-Damlama district; populations in Antalya-Akseki-Çambeleni, Isparta, Eğirdir-Aşağıgökdere, Osmaniye-Yarpuz-Yemşen; populations in Adana Karaisalı-Damlama and Antalya-Akseki-Küser district, Antalya Kemer and Kumluca, Isparta, Eğirdir-Aşağıgökdere, Kahramanmaraş Andırın-Efirağızlı, Mersin-Gözne, Mersin-Gülnar, Mersin-Tarsus- Çamalanı, Tekir and Osmaniye-Yarpuz-Yemşen; populations in Antalya-Akseki-Küser and Isparta, Eğirdir-Aşağıgökdere, Kahramanmaraş Andırın-Efirağızlı and Mersin-Tarsus; populations in Mersin-Tarsus Çamalanı-Tekir and Isparta, Eğirdir-Aşağıgökdere and Mersin-Gözne, populations in Osmaniye-Yarpuz-Yemşen and Isparta, Eğirdir-Aşağıgökdere, Kahramanmaraş Andırın-Efirağızlı, Mersin-Gülnar and Mersin-Tarsus. The topologies of NJ and ML trees and negative FST values indicated that *O. erosus* could be distributed in quite long distances.

D and FS test results of *O. erosus* showed that the populations in Antalya- Kemer and Antalya-Kumluca, Kahramanmaraş-Andırın Efirağızlı, Mersin-Gülnar and Mersin-Tarsus had an enlargement in the past. Furthermore, the sum of squared differences (SSD) and Raggedness index (Hri) results revealed that the populations only in Antalya-Kemer, Antalya-Kumluca and Mersin-Gülnar had enlargement.

Conclusion

Knowing of the genetic variations of bark beetles at local and regional level will provide highly useful data for forest management. The findings of this study, in which the genetic variations of *Orthotomicus erosus* that damaged pine trees including primarily the Brutian pine in the Mediterranean Region were analysed, show that there

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were not significant within population genetic variations or differences. These findings indicate that the concerned harmful bark beetles are highly mobile.

It was found that individuals of *O. erosus* that can feed on different pine species in the Mediterranean Region did not have any genetic variations and different populations were not disconnected and isolated geographically. This indicates that change of host does not result in dependence on a specific host for *O. erosus*; therefore, there is no barrier before the distribution of this species.

O. erosus does not have any sub-species. On the other hand, there is a need for further studies to explore if *O. erosus* depends on a specific geographical location and specific host species across Turkey considering its wide distribution in the country.

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ZOOPLANKTON COMMUNITY STRUCTURE IN SMALL PONDS IN RELATION TO FISH COMMUNITY AND ENVIRONMENTAL FACTORS

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Abstract. The studies were conducted in two small temperate mid-field ponds. The purpose of the research was to determine changes in the zooplankton communities in ponds with diverse ichthyofauna compositions and diverse values of environmental factors. Two research hypotheses were assumed. One pertained to the growth of the number and biomass of *Daphnia* and the loss of illoricate rotifers due to the top predator influence. The other one assumed that the top predators and the value of conductivity indirectly affect the diversity of zooplankton. We assumed that significant differences in the number of *Daphnia* between the ponds result from the regulation of the trophic web from the top down and bottom up, that affect the total zooplankton composition. In the small water bodies where *Daphnia* dominated among cladocerans, the highest numbers belong to loricate rotifers, e.g. *Brachionus sp.* and *Keratella sp.,* whereas in the ponds where the dominance among Cladocera was *Chydorus sphaericus,* illoricate rotifers were abundant, e.g. *Bdelloidea* and *Synchaeta sp.* We concluded that the presence of the top predator caused a significant increase in the species richness and in the biodiversity index for Cladocera. However, the biodiversity index for the whole zooplankton (determined mainly by small rotifers) decreased with the presence of top predator and increased with high conductivity.
Keywords: *biodiversity, biomanipulation, freshwater, plankton, ichthyofauna*

Introduction

The absence or presence of predator fish has a cascade effect on the whole trophic network of the water ecosystem (Hodgson, 2005). An increase in the biomass of predator fish entails a decrease in the biomass of plankton-feeding fish. The decrease in the number of plankton-feeding fish is followed by an increase in the biomass of filter-feeding zooplankton (Carpenter et al., 1985). However bottom up control which refers to the nutrients concentration in the environment and as a consequence food availability is well known to shape the assemblages of zooplankton (Gliwicz, 2002). Moreover, the taxonomic structure, body size and abundance of zooplankton are dependent on its taxonomic composition (Gliwicz and Siedlar, 1980), as well as on the season (Michael, 1969). It is believed that in small water bodies with diverse fish compositions, the mechanisms affecting the structure of the biocenoses from the top of the trophic network will prevail (Lampert and Sommer, 2001), which means that the biggest effect on the composition of species, abundance and biomass of individual zooplankton taxa will be exerted by fish, not by food availability.

The presence of a predator alters the behaviour of small cyprinids. This is manifested by their smaller activity and the preference of young cyprinids to hide in refuges, thanks

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to which daphnids can spend more time feeding in open waters (Romare and Hansson, 2003). Cladocerans such as *Daphnia* normally prevail in small water bodies when they are not randomly limited. Their prevalence results from the exclusion of smaller zooplankton taxa by competition (Diéguez and Gilbert, 2011). The presence of *Daphnia* in a pond may rapidly reduce the number of Rotifera and lead to taxonomic changes in their composition (Gilbert, 1988, 1989; Conde-Porcuna, 1998). *Daphnia* limit the survival of small illoricate rotifers, but has no effect on the survival of adult loricate rotifers (Diéguez and Gilbert, 2011). Despite numerous laboratory experiments, little attention is paid to taxonomic changes in natural small water bodies which may occur as a result of interaction of *Daphnia* with rotifers.

The majority of research in biodiversity and species richness of zooplankton pertained to the effect of trophic conditions on zooplankton (Dodson, 1992; Dodson et al., 2000; Leibold, 1999; Jeppesen et al., 2000; Declerck et al., 2007). Studies of the effect of daphnids on the biodiversity or species richness of zooplankton generally concern invasive species which could displace native crustacean species (Yan et al., 2002; Strecker et al., 2006). But maybe the native species of *Daphnia* have a negative effect on the biodiversity and species richness of rotifers and thus limit the biodiversity of the whole water body? Such a question may be put forward when using biomanipulation as a method for recultivation.

The purpose of this research was to determine changes in the structure of zooplankton communities in small mid-field ponds with diverse ichthyofauna composition and different environmental factors. The following hypotheses were assumed:

1) The presence of a top predator causes an increase in the number and biomass of *Daphnia* and loss of illoricate rotifers.
2) The top predator and the value of electrolytic conductivity correlate with the diversity of the zooplankton.

Methods and area of the research

Two mid-field ponds, Żelisławiec and Stare Czarnowo, with different taxonomical and quantitative composition of fish, located in the NW Poland, were chosen for studies (*Fig.1*). The studies were conducted in the spring, summer and autumn 2010 - 2014. The area of the Żelisławiec pond changed over the years and amounted to 0.91 to 0.64 ha and the area of the Stare Czarnowo pond changed from 0.82 to 0.40 ha. The ponds are similar as regards environmental conditions of drainage basin, which is entirely comprised of agricultural areas. Environmental conditions of drainage basin were determined on with the Corine Land Cover 2006 database. Agricultural areas encompass arable lands, permanent crops, meadows and pastures as well as mixed crop zones. Semi-natural areas are forests, semi-natural ecosystems and systems of shrub vegetation. Urban areas are urbanised areas, industrial areas and anthropogenised green areas. The drainage of the Żelisławiec pond was approximately 52 ha whereas the drainage of the Stare Czarnowo – 18 ha. The bed of two ponds was densely covered by macrophytes. Emerged and submerged vegetation occurred in both of the small water bodies. In the Żelisławiec, the emerged plants constituted 70% of the shoreline: *Typha latifolia* – 50% and *Phragmites australis* – 20%, whereas nymphaeid constituted 55% of the bed area: *Potamogeton natans* – 25%, *Persicaria amphibia* – 30%, and pleustophyte represent by *Lemna minor* – 5%. In the Stare Czarnowo pond, the emerged plants constituted 80% of the shoreline:

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Typha latifolia – 15% and *Phragmites australis* – 20%, *Glyceria maxima* – 45%, whereas the submerged plants constituted 80% of the area: *Ceratophyllum demersum* – 80%, and pleustophyte represent by *Lemna minor* – 5%.

The studies of fish fauna were conducted in summer 2010-2014. In order to determine the species composition of the fish fauna and the total body length of individual, the fish were caught using electric fish gear IUP 12 (Poland). In order to exclude a significant effect of the microhabitats on the shape of zooplankton communities, in each pond four different samples collection spots were selected. The samples were collected from the same place on each occasion. The results from the spots were averaged.

At each site 50 l of water were collected with a 5l bucket, which was filtered through plankton net with 25 μm mesh size. The samples were concentrated to 250 ml and were fixed in a 4 % formalin solution. Using the stirred total sample, ten sub-samples (3 mL) were pipetted into a glass Sedgewick-Rafter Counting Chamber. For identification, a Nikon Eclipse 50i microscope was used. Species were identified using the keys (Nogrady et al., 1993; Radwan, 2004; Dussart and Defaye, 2006; Rybak and Błędzki, 2010). In each sample, the body length of at least 30 individuals from each species was measured with the Pixelink Camera Kit 4.2. If the number of individuals representing a given species was lower than 30, the body lengths of all individuals were measured. The body length conversion to wet mass was made with the use of the tables (Ruttner-Kolisko, 1977; McCauley, 1984; Ejsmont-Karabin, 1998). Shannon diversity index and Sørensen similarity for zooplankton were calculated using the MVSP 3.22. The dominance structure was calculated from the mean values from all samplings. The level for dominance was established as 5% of the total abundance of zooplankton. A Mann-Whitney U-test was carried out to test for statistically significant differences, between sites, for environmental parameters, zooplankton communities and fish community. Spearman correlations were used to asses relationships between environmental parameters and between zooplankton and fish characteristics. Statistical analyses were performed using Statistica 10.

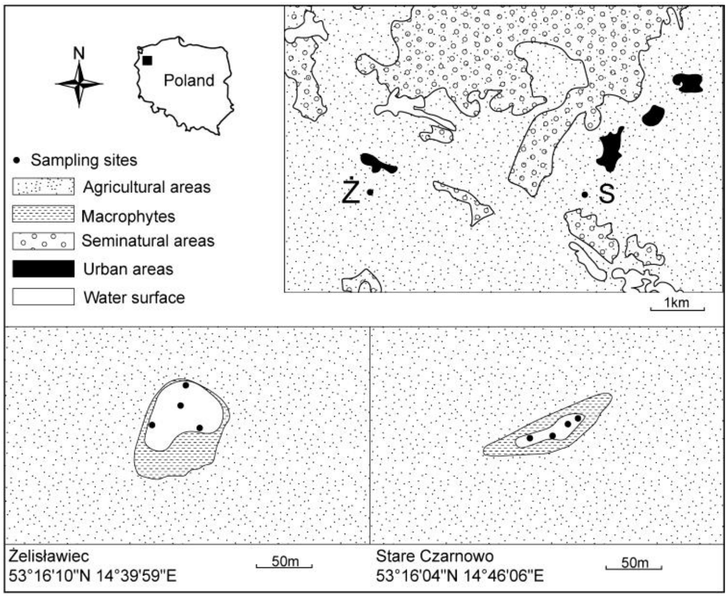

***Figure 1.** Map of the studied small water bodies and land use.*
Ż – Żelisławiec pond, S – Stare Czarnowo pond.

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Results

Abiotic factors

As regards abiotic factors in the Stare Czarnowo pond, the value of conductivity was nearly four times higher than in the Żelisławiec pond (P<0.05) (*Table 1*). A nearly twice as high concentration of dissolved oxygen was observed in the Żelisławiec pond than in the Stare Czarnowo pond (P<0.05).

Table 1. *Physical and chemical characteristic of pond and abundance, biomass, body size of Carassius sp in Stare Czarnowo and Żelisławiec (mean ±SD). Significant differences (Mann Whitney U test) * p <0.05, ** p <0.01, *** p <0.001.*

	n	Stare Czarnowo		Żelisławiec
pH	15	7.31±0.36		7.10±0.48
Conductivity (µS/cm^{-1})	15	406.7±69.8	***	113.8±36.4
Dissolved oxygen (mg/L^{-1})	15	2.61±1.26	**	4.38±1.71
Ammonium (mg/L^{-1})	15	0.34±0.79		0.16±0.12
Nitrate (mg/L^{-1})	15	0.14±0.16		0.11±0.17
Orthophosphate (mg/L^{-1})	15	0.72±0.5		0.72±0.5
Depth (cm)	15	114.1±43.3		103.7±34.6
Abundance *Carassius carassius*	5	1.0±2.2		5±3.3
Abundance *Carassius gibelio*	5	21.0±12.5		10.4±7.6
Biomass *Carassius carassius* (g)	5	42.6±95.2	*	1294.2±853.2
Biomass *Carassius gibelio* (g)	5	859.2±877.1		2247.2±1662.5
Size *Carassius carassius* (cm)	5	2.18±4.87	*	17.64±9.94
Size *Carassius gibelio* (cm)	5	9.56±6.25	*	23.14±3.34

Fish composition

Six fish species (*Carassius carassius, Carassius gibelio, Tinca tinca, Perca fluvitalis, Rutilus rutilus, Esox lucius*) were observed in the both small water bodies. All species occurred in the Żelisławiec whereas only *Carassius carassius* and *Carassius gibelio* were observed in Stare Czarnowo. *Carassius carassius* and *Carassius gibelio* in Żelisławiec were characterised by higher mean body mass and higher mean body length than in Stare Czarnowo (P>0.05) (*Table 1*).

Zooplankton taxonomic composition

Altogether 134 zooplankton taxa were observed in the studied ponds throughout the research period. In the Stare Czarnowo 112 taxa were revealed, 85 belonged to Rotifera, 12 Cladocera and 15 Copepoda. In The Żelisławiec 90 taxa were determined, 60 belonged to Rotifera, 16 Cladocera and 14 Copepoda. In Stare Czarnowo the zooplankton communities were dominated by *Bdelloidea* (18%), *Chydorus sphaericus* (37%) and *Eudiaptomus gracilis* (21%), whereas in Żelisławiec: *Keratella quadrata* (37%), *Daphnia longispina* (35%) and *Eudiaptomus gracilis* (27%) (*Table 2*). In Żelisławiec, the dominant rotifers were loricate species, whereas in Stare Czarnowo illoricate rotifers.

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***Table 2.** Zooplankton taxa of two studied ponds. + presence, - absence, H-mean abundance in group over 5%, D-dominant in mean abundance of the group.*

	Stare Czarnowo	Żelisławiec		Stare Czarnowo	Żelisławiec
Rotifera	85	60	*Polyarthra dolichoptera*	+	+
Anuraeopsis fissa	+	+	*Polyarthra remata*	-	+
Ascomorpha ecaudis	+	+	*Polyarthra longiremis*	+	+
Asplanchna priodonta	+	+	*Polyarthra vulgaris*	-	+
Brachionus angularis	+	H31%	*Rotaria rotatoria*	+	-
Brachionus budapestinensi	-	+	*Scaridium longicaudum*	+	-
Brachionus calyciflorus	+	+	*Squatinella mutica*	+	-
Brachionus leydigi leydigi	-	+	*Squatinella rostrum*	+	-
Brachionus quadridentatus	+	+	*Stephanoceros fimbriatus*	-	+
Brachionus rubens	+	H22%	*Stephanoceros* sp.	+	-
Brachionus urceolaris	-	+	*Synchaeta* sp.	H6%	+
Cephalodella catellina	+	-	*Synchaeta pectinata*	+	+
Cephalodella gibba	+	-	*Testudinella patina*	+	+
Cephalodella sp.	+	+	*Testudinella truncata*	+	-
Cephalodella sterea	+	-	*Trichocerca brachyura*	+	+
Cephalodella ventripes	+	-	*Trichocerca dixon-nuttalli*	+	+
Colotheca sp.	-	+	*Trichocerca iernis*	+	+
Colurella colurus	+	-	*Trichocerca insignis*	+	-
Colurella obtusa	+	-	*Trichocerca intermedia*	+	+
Colurella uncinata	+	+	*Trichocerca musculus*	+	-
Euchlanis deflexa	+	+	*Testudinella patina*	+	-
Euchlanis dilatata	+	+	*Trichocerca porcellus*	+	+
Euchlanis incisa	+	+	*Trichocerca pusilla*	-	+
Euchlanis lyra	+	+	*Trichocerca rattus*	+	+
Euchlanis oropha	+	-	*Trichocerca similis*	-	+
Filinia brachiata	-	+	*Trichocerca tenuior*	+	-
Filinia longiseta	+	+	*Trichocerca tigris*	+	-
Filinia maior	-	+	*Trichocerca weberi*	+	+
Filinia passa	-	+	*Trichotria pocillum*	+	+
Filinia terminalis	+	+	*Trichotria tetractis*	+	-
Hexarthra mira	-	+	Bdelloidea	D18%	+
Keratella cochlearis	+	H8%	**Cladocera**	12	16
Keratella cochlearis tecta	+	+	*Alona guttata*	+	+
Keratella hiemalis	+	+	*Alona rectangula*	+	+
Keratella irregularis	-	+	*Alonella nana*	-	+
Keratella quadrata	H6%	D37%	*Bosmina longirostris*	-	+
Keratella testudo	+	+	*Ceriodaphnia laticauda*	+	-
Keratella ticinensis	+	-	*Ceriodaphnia megops*	+	H7%
Lecane acus	+	-	*Ceriodaphnia pulchella*	-	+
Lecane arcuata	+	-	*Ceriodaphnia quadrangul*	+	+
Lecane bulla	+	+	*Chydorus sphaericus*	D37%	H16%
Lecane closterocerca	+	+	*Daphnia cucullata*	-	+
Lecane cornuta	+	-	*Daphnia longispina*	H11%	D35%
Lecane elsa	+	-	*Oxyurella tenuicaudis*	+	+
Lecane flexilis	+	-	*Peracantha truncata*	+	H12%
Lecane furcata	+	-	*Pleuroxus aduncus*	+	-
Lecane hamata	+	+	*Pleuroxus trigonelus*	-	+
Lecane ludwigii	+	+	*Scapholeberis mucronata*	-	H15%
Lecane luna	+	-	*Simocephalus expinosus*	H12%	+
Lecane lunaris	+	+	*Simocephalus vetulus*	H8%	+
Lecane quadridentata	+	-	**Copepoda**	15	14
Lecane quadridentata	+	-	*Cryptocyclops bicolor*	H9%	+
Lepadella acuminata	+	+	*Diacyclops bicuspidatus*	H13%	-
Lepadella heterodactyla	+	-	*Ectocyclops phaleratus*	+	-
Lepadella ovalis	+	+	*Eucyclops macruroides*	+	H7%
Lepadella patella	+	-	*Eucyclops macrurus*	H6%	H6%
Lepadella qinquecostata	+	-	*Eucyclops serrulatus*	H11%	H18%
Lepadella quadricarinata	+	-	*Eudiaptomus gracilis*	D21%	D27%
Lepadella rhomboides	+	+	*Macrocyclops albidus*	+	+
Lepadella triptera	+	-	*Macrocyclops distinctus*	-	+
Lophocharis oxysternoon	+	-	*Macrocyclops fuscus*	H7%	-
Monommata aequalis	+	+	*Megacyclops viridis*	+	+
Monommata longiseta	+	-	*Mesocyclops leuckarti*	+	H19%
Monommata maculata	+	-	*Paracyclops affinis*	+	H5%
Mytilina bisulcata	+	-	*Paracyclops poppei*	+	+
Mytilina mucronata	+	+	*Thermocylops crassus*	+	+
Mytilina ventralis	+	+	*Thermocylops oithonoides*	-	H5%
Mytilina trigona	+	-	Harpacticoida	H6%	+
Platyias quadricornis	+	+			

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The highest values of taxonomic similarity were recorded for the Żelisławiec pond in spring and summer (0.720), for Stare Czarnowo in spring and summer (0.646) and for both Stare Czarnowo and Żelisławiec in autumn (0.651) (*Table 3*).

Table 3. *Sørensen's similarity coefficient. S-Spring, Su-Summer, A-Autumn.*

		Stare Czarnowo S	Żelisławiec S	Stare Czarnowo Su	Żelisławiec Su	Stare Czarnowo A
Żelisławiec	S	0.582				
Stare Czarnowo	Su	0.667	0.534			
Żelisławiec	Su	0.519	0.72	0.531		
Stare Czarnowo	A	0.646	0.551	0.569	0.548	
Żelisławiec	A	0.585	0.661	0.569	0.613	0.651

The number of Rotifera taxa ($P<0.01$) (24 to 14), the number of zooplankton taxa ($P<0.05$) (31 to 22), the Rotifera biodiversity index ($P<0.01$) (2.07 to 1.34) and the zooplankton biodiversity index ($P<0.01$) (2.3 to 1.59) were significantly higher in Stare Czarnowo than in Żelisławiec.

Zooplankton abundance

In total zooplankton of both small water bodies rotifers had the highest percentage contribution in abundance. Moreover their percentage indicated growth from spring (Stare Czarnowo 53% and Żelisławiec 51%) to autumn, reaching the maximum of 75% in Stare Czarnowo and 91% in Żelisławiec (*Fig. 2*). In spring the percentage share of crustaceans in both ponds almost reached 50%. Moreover, in spring the proportion of Cladocera in the population was six times higher in Żelisławiec than in Stare Czarnowo. The density of *Daphnia* in Żelisławiec was over fourteen times higher than in Stare Czarnowo ($P<0.05$) (3.2 to 46.9 ind./dm^{-3}) (*Table 4*).

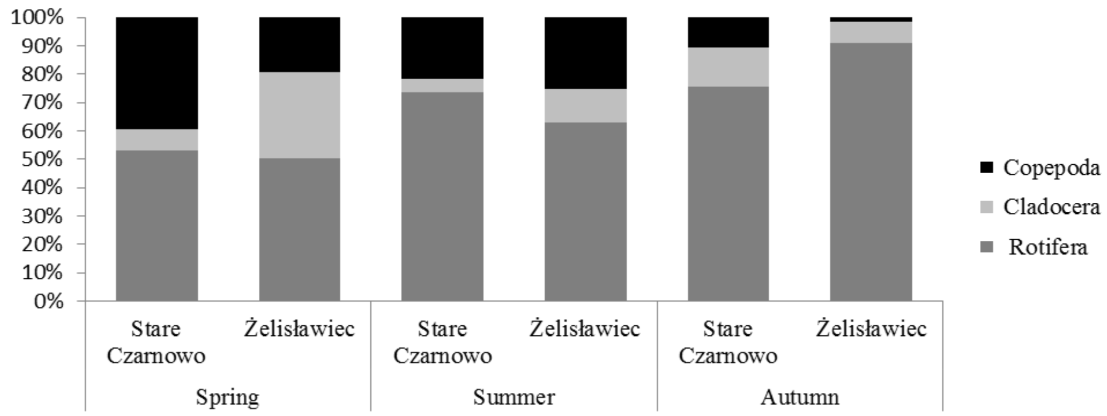

Figure 2. *Percentage of mean abundance of zooplankton in seasons.*

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Table 4. *Value of zooplankton factors (mean ±SD) in ponds. Significant differences (Mann Whitney U test) * p <0.05, ** p <0.01, *** p <0.001.*

	n	Stare Czarnowo		Żelisławiec
Abundance *Rotifera* (ind. L^{-1})	14	94.3±61.5		243.6±272.8
Abundance *Cladocera* (ind. L^{-1})	14	11.0±10.8		74.8±88.1
Abundance *Copepoda* (ind. L^{-1})	14	45.9±54.1		63.7±72.2
Abundance *Copepoda* (mature) (ind. L^{-1})	14	2.9±3.0		4.5±5.3
Abundance All zooplankton (ind. L^{-1})	14	156.8±108.1		358.5±320.9
Abundance *Daphnia sp.* (ind. L^{-1})	14	3.2±8.7	*	46.9±81.2
Biomass *Rotifera* (mg. L^{-1})	14	0.23±0.52		0.36±0.36
Biomass *Cladocera* (mg. L^{-1})	14	0.72±0.66		7.74±10.71
Biomass *Copepoda* (mg. L^{-1})	14	0.53±0.61		0.8±0.87
Biomass All zooplankton (mg. L^{-1})	14	1.3±1.05	*	8.89±11.05
Number of taxa *Rotifera*	14	24.0±9.1	**	14.9±5.8
Number of taxa *Cladocera*	14	4.1±2.5		5.3±3.5
Number of taxa *Copepoda*	14	3.0±2.1		2.4±2.1
Number of taxa All zooplankton	14	31.1±11.1	*	22.6±8.6
Shannon Index *Rotifera*	14	2.07±0.53	**	1.34±0.63
Shannon Index *Cladocera*	14	0.83±0.54		0.79±0.56
Shannon Index *Copepoda*	14	0.81±0.60		0.54±0.53
Shannon Index All zooplankton	14	2.3±0.51	**	1.59±0.64

Zooplankton biomass

In both small water bodies, crustaceans dominated in the zooplankton biomass (*Fig. 3*). In Stare Czarnowo the percentage of Cladocera biomass ranged from 42% in spring to 61% in summer and 50% in autumn. In Żelisławiec, the percentage of Cladocera was higher and amounted to 84%, 91% and 86% from spring to autumn respectively. The percentage of Rotifera biomass in Stare Czarnowo increased from spring (6%) to autumn (34%) whereas in Żelisławiec it remained on a low level and did not exceed 8%. An almost seven times higher mean zooplankton biomass was observed in Żelisławiec than in Stare Czarnowo (P<0.05) (1.30 to 8.89 mg/dm^{-3}).

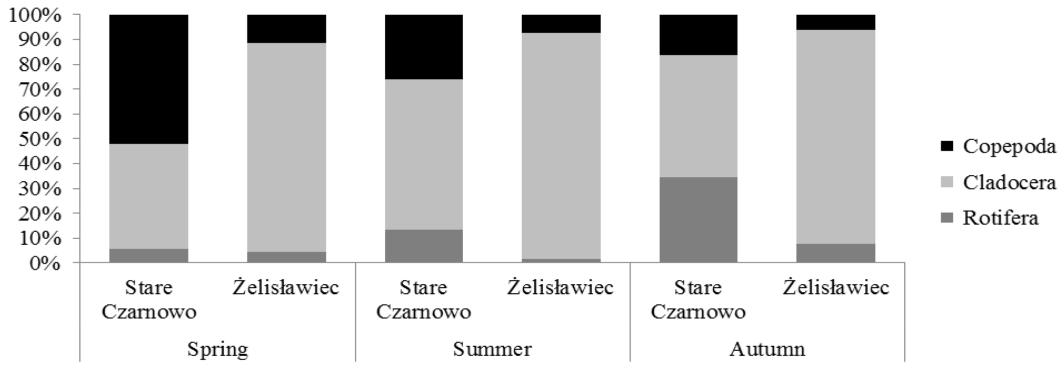

Figure 3. *Percentage of mean biomass of zooplankton in seasons.*

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Correlation between zooplankton composition and environmental factors

The simple correlation indicated that eleven environmental variables correlated significantly with the values of fifteen variables of zooplankton ($p<0.05$) (*Table 5*). The abiotic parameter which correlated the most often with the structures of zooplankton was depth. Further variables correlated with these structures were the content of dissolved oxygen and the value of conductivity. The fish predominantly displayed a significant correlation with Rotifera structures and Cladocera biodiversity index. In the former case, the number of Rotifera taxa was negatively correlated with the number of pike, Prussian carp biomass and pike biomass but was positively correlated with sizes of the crucian carp. In the latter case, the Cladocera biodiversity Index was negatively correlated with the number of Prussian carp and positively correlated with the number of pike.

Table 5. *Significant correlations (p <0.05) of Spearman analysis between the factors of zooplankton and environmental variables.*

	Abundance *Rotifera*	Abundance *Cladocera*	Abundance *Copepoda*	Abundance *Copepoda* (mature)	Abundance all zooplankton	Abundance *Daphnia*	Biomass *Cladocera*	Biomass *Copepoda*	Biomass All zooplankton	Number of taxa *Rotifera*	Number of taxa *Copepoda*	Number of taxa all zooplankton	Shannon Index *Rotifera*	Shannon Index *Cladocera*	Shannon Index all zooplankton
Abundance *Carassius gibelio*														-0.72	
Abundance *Esox lucius*										-0.83				0.74	
Biomass *Carassius carassius*										-0.89			-0.79		
Biomass *Carassius gibelio*									0.79						
Biomass *Esox lucius*										-0.72					
Size *Carassius carassius*										-0.83					
Size *Esox lucius*													-0.79		-0.76
Conductivity		-0.42				-0.54	-0.40		-0.52	0.47			0.44		0.38
Dissolved oxygen		0.39				0.41	0.48		0.57						
Phosphate			-0.38									-0.43			
Depth	0.40		0.49	0.53	0.46	0.53		0.44	0.41		0.47				

Discussion

Results of analyzed data show that the presence of a top predator causes direct and indirect changes on structure of zooplankton namely increase in the number and biomass of *Daphnia* and loss of illoricate rotifers which affect species richness and biodiversity of zooplankton. The results of our research and analysis of the results of other authors (Carpenter et al., 1985; Jeppesen et al., 2000; Hodgson, 2005; Declerck et al., 2007; Diéguez and Gilbert, 2011) confirming our hypotheses.

The taxonomic and quantitative composition of zooplankton of the studied ponds is similar to those observed by other authors in similar small water bodies (Radwan, 2004; Segers, 2008). Distinct prevalence of rotifers over crustaceans as regards the number of taxa and their number has been demonstrated numerous times (Karabin, 1985; Herzig, 1987; Kuczyńska-Kippen, 2009).

The studied ponds differed in dominants in the Rotifera and Cladocera groups. A large number of loricate rotifers in Żelisławiec may directly result from the prevalence of specific Cladocera taxa, particularly *Daphnia*. In fishless ponds *Daphnia* may often

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severely limit the abundance of small rotifers by mechanical interference (ingestion and damage after rejection) (Diéguez and Gilbert, 2011). They revealed that adult *Brachionus sp.* avoided fatal consequences of interference with *Daphnia*. This is indicated by the fact that where *Daphnia* prevail, the biggest numbers are demonstrated by loricate rotifers, but where the dominance among Cladocera was *Chydorus sphaericus,* then illoricate rotifers were abundant *(Bdelloidea* and *Synchaeta sp.)*. It is presumed that *Daphnia* also had some effect on the lower number of zooplankton taxa in Żelisławiec than in Stare Czarnowo, including as many as 25 fewer Rotifera taxa.

Differences in the zooplankton communities between spring and summer were observed in both ponds, which may indicate stability in the species composition in both small water bodies. A significantly higher number and biomass of Cladocera (mainly *Daphnia*) in Żelisławiec (with top predator) than in Stare Czarnowo seems to have limited the number of Rotifera taxa and taxa of small cladocerans. A poor population of cladocerans as Dapnia in spring in Stare Czarnowo resulted from the high pressure from small cyprinids, which caused better conditions for development of small crustaceans and illoricate rotifers. In spring and summer, when cyprinids hatch, the pressure on cladocerans is the largest. Zooplankton abundance declined greatly after the peak in fish larval abundance (Welker, 1994). Therefore, in spring and in summer, we could observe different zooplankton structures in both ponds. In autumn, however, high similarity between the two ponds was observed. Perhaps, the autumn fry was too large to significantly reduce the populations of larger plankters in the pond where pike was absent. Fluctuations in zooplankton populations can occur both spatially and temporally and may be caused, in part, by predation from planktivorous fishes (Welker, 1994).

The abundance domination of Rotifera is typical of such small mid-field water ecosystems (Kuczyńska-Kippen, 2009; Mieczan et al., 2016). However, the domination was not evenly distributed in different seasons, as mentioned above. The increase Rotifera percentage in the abundance of zooplankton from summer to autumn may pertain to the feeding on crustacean by fry and consequently increasing the density of rotifers. However, as pointed out above, the proportion of rotifers in the number of zooplankton was smaller in the pond with pike than in the pond without it. The value of zooplankton biomass in both ponds can be justified in a similar manner. Generally the zooplankton biomass is dominated by cladocerans due to their larger sizes, predominantly, when there is no factor which would limit them (Carpenter et al., 1985). Simultaneously, two aspects affected higher biomass in the pond with pike: the altered behaviour of Prussian carp which resulted in reduced pressure on cladocerans and a reduced number of small cyprinids, as well as the reduction of rotifers by the filtration mechanism of large ones (Diéguez and Gilbert, 2011).

Changes in the density of large piscivorous fish results in changes in density, species composition, and behaviour of zooplanktivorous fish. Planktivorous fish select the largest available prey and can rapidly reduce the density of zooplankters (Carpenter et al., 1985; Gliwicz, 2002). So, we believe that the high mean sizes of the Prussian carp in Żelisławiec can be justified by the occurrence of a predator which reduced the number of young individuals of the Prussian carp. In Stare Czarnowo, where pike was absent, the Prussian carp had smaller mean sizes and their number was higher, which might have contributed to the reduction of some zooplankton taxa. Significant differences in the number of *Daphnia* between the small water bodies result from the regulation of the trophic network from the top and affect the whole zooplankton structure. This is a common pattern of biomanipulation which is used as one of the methods for lake

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recultivation (Jeppesen et al., 2007). Top-predators are not always associated with biodiversity benefits. On the basis of research of large land predators, it was proved that the top predator may have a negative effect on endemic species or may reduce other precious smaller species (Allen et al., 2012; Duffy et al., 2007). Duffy et al. (2007) put the question. "What are the community and ecosystem-level consequences of biodiversity loss?". In the case of the small water ecosystems we analysed, the absence of a predator and, simultaneously, the lack of one trophic level cause reorganisation of the zooplankton structure. It also has a different effect on various groups of planktonic organisms, which will be discussed further.

The strongest negative correlations between the number of Rotifera taxa and the biomass of the Prussian carp and its sizes can be justified by the fact that larger Prussian carps beginning to feed on macro-invertebrates discontinue feeding on cladocerans (Tsoumani et al., 2006) which led to the domination of the largest possible plankters which were not limited by predators. Similar significant correlations were observed between the biodiversity index and the number of the Prussian carp. It is common knowledge that young cyprinids feed on crustaceans and food selectivity (Gliwicz, 2002) may lead to displacement of some species. Therefore, a large number of a predator which limited small plankton-feeding Prussian carp caused a significant increase in the Cladocera biodiversity index. The biodiversity index for the total zooplankton was negatively correlated with total length of pike, which results from the fact that the overall zooplankton biodiversity is mainly shaped by Rotifera, due to their domination in density and the number of taxa. Links between biodiversity and ecosystem function provide compelling reasons for conserving maximal numbers of species in ecosystems (McGrady-Steed et al., 1997). It also has to be noted critically that a different species composition of submerged macrophytes in both ponds could have affected species diversity and biodiversity (Schriver et al., 1997).

The abiotic parameter which correlated significantly positively with the biggest number of taxa was depth. We believe that higher abundance and biomass in high water level result from surface runoff that promote a high re-suspension of sediments into the water column. This hypothesis requires further research.

The zooplankton composition in both ponds could have differentiated in the values of conductivity, which differed significantly the small water bodies. According to Sousa et al. (2008), high values of conductivity is often correlated with high trophy status and among many of the environmental variables, conductivity significantly explains the principal variations in the species composition of the zooplankton community. Similarly, Żurek (1983) did not reveal any significant correlations between conductivity and zooplankton communities. However, in the present study effect of conductivity on the number of zooplankton, species richness and biodiversity index was observed. Bērziņš and Pejler (1989) suggest that species as *Brachionus sp*. stand out for its great tolerance to high conductivity. Diverse value of conductivity in studied ponds were a factor that supports different zooplankton community that we think was mainly shaped by fish community.

Another important variable differentiating both ponds was the concentration of dissolved oxygen. Zooplankton can tolerate lower oxygen concentrations than fish and may use oxygen gradients as refuges against predation (Horppila et al., 2000). Low dissolved oxygen concentration has little influence on zooplankton (Yang et al., 2012). In our study in pond with higher oxygen concentration and lower conductivity (Żelisławiec) we determined low taxa richness and biodiversity of zooplankton. Even

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though oxygen concentration was high no positive effect on taxa richness and biodiversity of zooplankton was observed. We assume that fish community in this study has been a leading factor. Moreover, higher oxygen concentration and lower values of conductivity in Stare Czarnowo may have been caused by the presence of pike. Many authors describing biomanipulation as a target effect demonstrate improvement of physico-chemical conditions (Hodgson, 2005; Jeppesen et al., 2007; Eriksson et al., 2009) which may be related to an increase in dissolved oxygen concentration and a decrease in conductivity values, which were observed in the ponds.

Summary

Changes in density of large piscicorous fish result in changes in density, species composition, and the behaviour of the zooplanktivorous that select largest available prey and can rapidly reduce the density of zooplankters. In the pond with pike, crucian carps were characterised by bigger sizes and a smaller number of individuals, which was attributed to the lower pressure on cladocerans. Significant differences in the abundance of *Daphnia* between the small water bodies result from the regulation of the trophic web from the top and affect the whole zooplankton structure. However bottom up control which refers to the nutrients concentration in the environment and as consequence food availability shape the assemblages of zooplankton. From the other hand high conductivity values could be a factor that promotes some species e.g. *Brachionus sp*. In ponds with top predator, large *Daphnia* severely limited the abundance of small rotifers by mechanical interference (ingestion and damage after rejection). In the pond where *Daphnia* dominated among cladocerans, the highest numbers belong to loricate rotifers, e.g. *Brachionus sp.* and *Keratella sp.,* whereas in the pond where the dominance among Cladocera was *Chydorus sphaericus,* illoricate rotifers were abundant, e.g. *Bdelloidea* and *Synchaeta sp.* The presence of the top predator caused a significant increase in the species richness and in the biodiversity index for Cladocera but higher conductivity values supports higher species richness and biodiversity of all zooplankton. The overall zooplankton biodiversity is shaped by Rotifera which may be limited by large cladocerans, due to the top effect of the top predator on the trophic network.

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IRON ORE WASTE CLASSIFICATION ACCORDING TO UNEP GUIDELINES (CASE STUDY: GOLGOHAR MINING AND INDUSTRIAL COMPLEX IN SIRJAN, IRAN)

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Abstract. Population growth has prompted governments to encourage industrial development in order to improve the welfare of their citizens. This process increases the deposition of human and industrial waste in the environment. The consequences of this have produced a serious need to implement appropriate management strategies in order to reduce and control the effect of the accumulation of such waste. The current study was undertaken to compile information about the status of iron ore waste produced by the Golgohar Mining and Industrial Complex in Sirjan, Iran, in 2015 by observation, field visits, interviews with officials and experts of the unit and the use of questionnaires and technical documentation. The industrial waste was classified using UNEP guidelines and guidelines for proper management of waste have been presented. The results of data analysis regarding the quantity and quality of mining and industrial waste of Golgohar Mining and Industrial Complex showed that about 24 million tons of solid industrial waste is produced annually. A large percentage comprises hazardous waste according to UNEP classifications and include rock and soil tailings from processing, dry and wet tailings. These are classified into group E as high volume and low-risk waste and in group A as mining waste containing heavy metals. The presence of heavy metals increases the importance of controlling and managing this waste.
Keywords: *hazardous waste, solid waste, heavy metals, United Nations Environment Programme*

Introduction

Since the development of mining industry, mining exploitation activities have enhanced solid wastes production and induced increasingly grievous destruction on the eco-environment. Waste rock, tailings and other solid waste are the largest industrial solid waste generated in the process of exploitation of mineral resources (Lu and Cai, 2012). Mining activity leaves behind a great variety of waste materials which often contain high concentrations of toxic elements (András et al., 2012).There exist a large number of issues such as stockpiled tailings resources, environment, safety and land, etc. Since there is a technology gap between different periods of smelt, a great deal of valuable resources is being retained in the tailings, and if these tailings resources cannot be integrated in the recycling process, it will cause a huge waste on the enviroment (Yang et al., 2011). Mining wastes are referred to as those wastes produced during

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mining operations in the form of returned load, mining overburden or tailings (BHP Billiton Mitsubishi Alliance, 2009). In general, mine tailings are mechanically, physically, chemically and biologically deficient (Vega et al., 2006), characterized by instability and limited cohesion, with low contents of nutrients and organic matter and high levels of heavy metals (He et al., 2005).Opencast mining activities have a serious environmental impact on soils and water streams, generating millions of tons of mine tailings (Bhattacharya et al., 2006). With the rapid development of iron and steel industry, the proportion of iron ore tailings in industrial solid waste is rapidly increasing. According to incomplete statistics, the total amount of cumulative iron tailings is nearly $2* 10^9$ tons (Wang et al., 2009).

On the other hand, mining and smelting of metal ores have increased the occurrence and spread of heavy metals contamination in the soil, especially open pit mining which produces million tons of mine tailings, which have serious environmental impact on soil and water flow (Bhattacharya et al., 2006). The contamination of soil by heavy metals is one of the major environmental problems facing many countries around the world, considering their almost indefinite persistence in the environment and the danger they pose (Ding et al., 2013). Therefore tailings are environmentally problematic in that they are natural (crushed) particles impregnated with the chemicals used in mining. Tailings which contain iron sulfide also contain heavy metals such as antimony, arsenic, beryllium, cadmium, lead, nickel, selenium, silver and zinc. These tailings can cause the environment to produce acidic drainage in the presence of oxygen and water (Balvardi, 2010). Among other environmental concerns in relation to tailings from the mining of iron ore is the presence of fibrous forms of mineral silicates belonging to the serpentine and amphibole groups of rock-forming minerals, including asbestos in tailings (Kesler, 1994).Incidents of poor waste management practice are amongst the most conspicuous features of the global minerals industry. Tailings spills, dam failures, seepage, unrehabilitated sites and cases of direct discharge into water ways can result in severe and long-term environmental and social consequences (Van Zyl, 1993; ICME and UNEP, 1998; Hart, 2007; Franks, 2007; Spitz and Trudinger, 2009; Fourie, 2009).

Mining and industrial waste, if not properly controlled, will create problems for industrial units. They occupy space, can cause injury to employees, create odors and attract insects and stray animals. Dumping of debris on surrounding land can cause complaints and enforcement by regulatory bodies. Such materials are usually produced in large quantities, which will increase the cost of storage, handling and disposal; however, it can also make recycling economically justified (Habibinejad, 2000). Studies in China, India, Russia and Spain have shown the widespread use of iron mine tailings to be economically feasible and, in many cases, profitable. It reduces the volume of waste and, under optimal management, the regional environmental situation can be improved. Use of the proposed operation and management strategies can also compensate for or reduce the cost of production (Zhang et al., 2006).

In India approximately 10 – 15 % of the iron ore mined is unutilized, even now, and is discarded as waste/tailings due to lack of cost effective technology in extracting low grade ores. Because of this huge piles of mine wastes are found in and around mine areas (Ahmari and Zhang, 2012). However, some of these waste materials possess potential characteristics, which can be tapped for various uses (Hussain, 1995; Water, 2001; Menezes and D‘Souza, 2004; Kumar, 1998). Hammond (1998) in his study critically reviewed the usage of mining waste as building material. He identified many mining wastes as concrete aggregates and pigments for paints. Das et al. (2000)

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developed new techniques for converting iron ore tailings into value added products such as ceramic floor and wall tiles for building application. It was proved that such tiles have high strength and hardness compared to conventional tiles. The investigation also revealed other benefits like energy economy and lower production costs, which is also strengthened by the study carried out by Reddy (2004) and Zhang et al. (2006).

Mining waste is environmentally hazardous because of the large volume produced and the presence of chemical reagents. Heavy metals can be found in some types of mining waste, indicating the need for proper management of its disposal.

The costs related to the control and management of hazardous waste are higher than for other waste and its mismanagement can incur heavy fines. It is very important for a manufacturing unit to operate economically (Habibinejad, 2000); thus, all waste management plans must consider the economic aspects as of equal importance with the environmental issue.

Literature Review

Decision-making on waste infrastructures is difficult because waste management is a complex, politically loaded and emotionally charged issue that is neither well structured, nor well understood. While sustainability is the ultimate goal of the EU environmental policy, there is no commonly accepted approach for its realization (Verhoef et al., 2006).

The first step in waste management is to identify and classify waste according to given standards and laws. The first laws and regulations pertaining to hazardous waste management were implemented by European common market countries in 1980. The United Nations Environment Programme (UNEP) established the principles of environmental management of hazardous waste in 1987. The Basel Convention was adopted by 35 participating countries in Switzerland in 1989 to control trans boundary movement of hazardous waste. At present, 105 countries, including Iran, have joined the treaty since September 1992 (Binavapoure, 2010). The waste law was passed in Iran in 2004. The law consists of 23 articles and waste has been classified into five categories, including those of hazardous(or special) waste. Hazardous waste refers toall waste that requires special handing due to the presence of at least one of dangerous property (toxicity, pathogenicity, possibly explosive or ignitable and corrosive). Similar to medical waste and some general waste, industrial, mining and agricultural waste that requires special management is classified as special or hazardous (Office of Legal and Parliamentary Affairs, 2004).

Different methods have been used for the classification of waste, including those based on how waste is produced, its detrimental effects, the degree of risk and the physical state (Abdoli et al., 2010). Each method of identification and classification of hazardous waste has advantages and disadvantages. The use of lists and directories is a relatively simple method for the identification of hazardous waste. The most important directories and classifications include the US Environmental Protection Agency (USEPA) quad list, UNEP classification and Basel Convention classification (Abdoli et al., 2010). In Iran, classification of industrial waste is often done based on these three classifications.

Sabzalipour et al. (2007) studied the feasibility of industrial waste minimization in Bandar Imam Petrochemical Complex and first identified the waste, its quantity and quality and then classified it according to UNEP guidelines (Sabzalipour et al., 2007).

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Namdari and Turkian (2010), studied management of waste and chemicals used in Asaluyeh Pardis Petrochemical Company and classified them according to the Resource Conservation and Recovery Act (RCRA) and Basel Convention (Namdari and Torkian, 2010). The present study was undertaken to identify all industrial waste from the Golgohar Mining and Industrial Complex in Sirjan, Iran. Beside waste identification, the quality and quantity was determined and the type of waste was classified according to UNEP guidelines.

Materials and Methods

Study area

Golgohar Mining and Industrial Complex (29°7′5″N and 55°19′5″E) (*Figure 1*) is located 55 km South West of Sirjan city in Kerman province, south of Iran. According to Iran's National Census in 2006, the population of Sirjan is 167,014, scattered in 40,605 families. The study area is located at an altitude of 1730 m above the sea level; it is situated in a depression between the southern Zagros Mountains to the west and the Kuh-e Bidkhan massif to the east (Sirjan county, 2015). Golgohar Mining and Industrial Complex is the main source of revenue for the city. It meets 30% of the needs of steel fatories in Iran. Based on the method of De martonne, drought index was 39/6, and based on this classification, the climate of the study area is dry (Kousha Madan Consulting company, 2014).

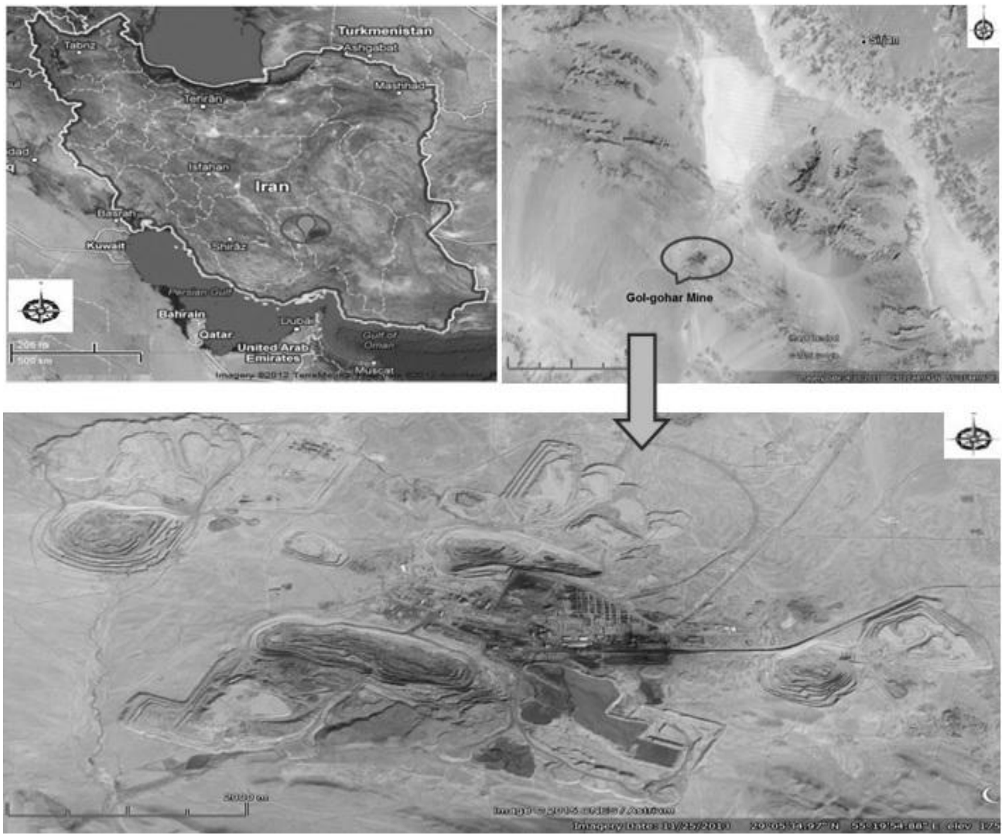

Figure 1. *Study area in Sirjan County, Kerman Province, Iran*

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Golgohar Mining and Industrial Complex comprises four plants, three of which are involved with ore beneficiation and concentration. These are the magnetite processing, hematite recovery and desulfurization and polycom processing plants. The fourth plant is the pelletizing plant, which is responsible for the agglomeration of concentrates produced by the other three plants. Each plant produces different types of waste according to their process and the raw material and additives used.

Methods

To carry out this research, field visits and interviews with the managers and owners of industry were carried out and a questionnaire containing 29 questions was developed and completed. To obtain accurate and real date, information was also obtained from annual reports of the environmental unit and technical documents from the plants and MSDS instructions and executive program reports on waste management were disclosed. This neutralized the subjectivity of the manager responses, which can be a prevalent problem on similar research efforts.

Ultimately, UNEP guidelines were used in the pluralization of the information obtained and classification of waste. Recommendations and management strategies were then presented using the results. The method used in this study consisted of four parts:

- Part I: Library research;
- Part II: Field visits;
- Part III: Questionnaire preparation;
- Part IV: Classification of waste in accordance with UNEP guidelines and provision of appropriate management strategies.

In the first part, the method applied involved gathering the most recent statistics and background information using the resources available in libraries, scientific and research centers, Golgohar Mining and Industrial Complex and internet searches in the fields of mining, environment and waste management. In the second part, in order to become more familiar with the complex's performance, field visits and interviews were conducted with managers of industry, then producer points and sources of industrial waste were identified through field visits. In the third part, the quantity, nature and mode of industrial waste were identified through the questionnaires.

In the fourth part, after analysis of the collected data, an attempt was made to classify the waste based on the type produced and according to the UNEP guidelines. Thereafter, appropriate strategies for waste management were provided. This study was based on the information obtained from the exploitation and processing units of Golgohar Mining and Industrial Complex and focused on studying the quality and quantity of industrial waste and classifying it according to international standards.

Classification of waste

From the perspective of UNEP, hazardous waste is defined as waste materials that is either solid, sludge, liquid or gas in the tank, except for radioactive materials, which have chemical activity, toxicity, explosive and corrosive properties or other characteristics, and has been shown to pose detrimental effect on human health or the environment either by itself or when mixed with other materials (LaGregaet al., 2001).
UNEP classification is based on the type of waste, industry or process involved in the production of hazardous waste. One advantage of this classification is that it can largely

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make the environmental administrators and managers aware of the types of industry and processes that must be controlled. In this classification, the type of waste material, industry or process and the abbreviated titles of the groups are presented in table form. The waste was divided into six groups (Abdoli et al., 2010) as shown in *Table 1* according to UNEP guidelines.

Table 1. *Classification of waste according to UNEP*

<table>
<tr><th colspan="2">Group A</th><th>Nature of the waste</th><th>Group B</th><th colspan="2">Group C</th><th>Nature of the waste</th><th>Group D</th><th>Group E</th><th colspan="2">Group F</th><th>Nature of the waste</th></tr>
<tr><td rowspan="6">1-Mining waste</td><td>1-1</td><td>Acids and alkali</td><td rowspan="6">2-Oily waste</td><td rowspan="6">3-Organic Waste</td><td>3-1</td><td>Used halogenated solvents</td><td rowspan="6">4-Putrescible organic materials</td><td rowspan="6">5-High volume and low-risk waste</td><td rowspan="6">6-Miscellaneous waste</td><td rowspan="2">6-1</td><td rowspan="2">Infectious waste</td></tr>
<tr><td>1-2</td><td>Wastes containing Cyanide</td><td>3-2</td><td>Used non-halogenated solvents</td></tr>
<tr><td rowspan="2">1-3</td><td rowspan="2">Dissolved and sludges containing heavy metals</td><td>3-3</td><td>Wastes containing PCB</td><td rowspan="2">6-2</td><td rowspan="2">Laboratory Waste</td></tr>
<tr><td>3-4</td><td>paints and resins waste</td></tr>
<tr><td>1-4</td><td>Wastes containing Asbestos</td><td>3-5</td><td>Pesticide waste</td><td rowspan="2">6-3</td><td rowspan="2">Explosive waste</td></tr>
<tr><td>1-5</td><td>uncertain Solid remains</td><td>3-6</td><td>Chemical organic materials</td></tr>
</table>

Mining/smelting is one of the main sources of heavy metal pollution in the environment, and the tailings generally contain higher concentration of heavy metals. Therefore, several researches have been conducted on different metal mining tails and surroundings to determine the behavior of the heavy metals (Rodrguez et al., 2009; Romero et al., 2007; Moncur et al., 2005; Lowry et al., 2004).

The findings indicates that mine tailings can be classified using the UNEP method of classification based on the presence of heavy metals, as well as its high volume.

Results

The information obtained from the questionnaire regarding the qualitative and quantitative assessment of waste from Golgohar, interviews with managers and experts and a review of reports and technical documents helped determine the type of waste produced in the mining region. It comprised tailings of rock and soil from ore extraction, dry and wet tailings from magnetic separation, effluent from flotation cells containing tailings and reagents used in flotation and oil and grease used on equipment, in addition to worn-out components and equipment.

Industrial waste of different types can be classified according to different criteria. Some researchers have classified industrial waste based on their generation source, while others have classified them based on their specifications. Waste can also be classified according to its internal components. What is important for classification is to facilitate the identification and management of waste. Industrial waste can be classified as either hazardous or non-hazardous.

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The three types of waste identified were analyzed for seven elements (heavy metals) with respect to the degree of danger (degree of hazard of each type of waste and the most significantly hazardous by type). The seven elements were chosen using the toxicity characteristic leaching procedure (TCLP) approved by the USEPA to determine the hazardous waste by testing for 40 elements in and the composition of waste. Seven elements that were measurable by Iranian laboratory methods were selected of the 40 TCLP elements and tested to determine the hazardous nature of each type of tailing and its degree of danger. *Table 2* shows that results for three types of tailings for the seven elements: silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr), lead (Pb), vanadium (V) and zinc (Zn) was done (*Table 2*).

Table 2. *The amount of heavy metals in each type of tailing*

Tailing type	Ag (ppm)	As (ppm)	Cd (ppm)	Cr (ppm)	Pb (ppm)	V (ppm)	Zn (ppm)
Worthless tailings rock and soil	< 1	5	< 1	41.81	32.36	82	56.72
Dry tailings	< 1	9.2	< 1	27.2	14.6	63.8	39.2
Wet tailings	< 1	6.5	< 1	33.66	32.75	206.75	69.08

The results indicate that the presence of heavy metals, especially Cr, Pb, V and Zn were above normal. In addition to being classified as hazardous according to the UNEP, their large volume require environmental measures to optimize the management of these types of waste.

The wet tailings had the highest amount of Pb and Zn and a significantly higher amount of V. Dry tailings contained the highest amount of As and rock and soil tailings contained the highest amount of Cr.

Hazardous waste of Golgohar Mining and Industrial Complex

- Worthless tailings rock and soilfor processing are classified as group E "**High volume and low-risk waste"** according to UNEP's classification.
- Dry tailings are classified as group A "**Mining waste containing heavy metals"** according to UNEP's classification.
- Wet tailings are classified as group A "**Mining waste containing heavy metals"** according to UNEP's classification.
- Used oils, including compressors and mills oil,etc are classified as group B "**Oily waste"** according to UNEP's classification.
- Burned grease is classified as group B "**Oily waste"** according to UNEP's classification.
- Used activated carbon is classified as group C "**Organic Waste"** according to UNEP's classification.

Any type of waste with at least one dangerous property can be called hazardous waste. These include chemical activity, toxicity, pathogenicity, explosion or ignition creation, corrosion and any others that are risky or likely to cause harm to human health or the environment, either by itself or when mixed with other materials.

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Non-hazardous waste of Golgohar Mining and Industrial Complex

Any type of waste that is not classified into groups A, B, C, D, E and F is known as non-hazardous waste. Accordingly, non-hazardous waste would include used activated alumina, molecularsieves, worn balls and rods from mills, steel liners from mills, rubber liners and parts of mills, abrasive parts of pumps, filter and cleaning cloth, wire and cotton strips, plastic sectors, lifter bars, impact bars, shell plates, wooden cartons, boxes and pallets, great bars (metal parts of the pallet floor) and steel pallets.

Classification of hazardous waste according to their characteristics

Waste is called hazardous if it possesses corrosive, explosive or ignitable or toxic properties or shows chemical activity. Given the importance of this issue, after identifying the waste in the study area, it was characterized and classified. The results are shown in *Table 3*.

Table 3. *Classification of GolGohar Mining and Industrial Complex's wastes based on UNEP's method*

Order index	**Waste**	**Hazardous waste's classification**						**Specifications**				**Non-hazardous waste**
		A	***B***	***C***	***D***	***E***	***F***	***Corrosive***	***Ignition creation***	***High affinity***	***Toxicity***	
1	Worthless tailings rock and soil					*					*	
2	Dry tailings	*									*	
3	Wet tailings	*									*	
4	Used oils		*						*			
5	Burned grease		*						*			
6	Used activated carbon			*					*			
7	Used activated alumina											*
8	Molecularsive											*
9	Worn balls and rods of mills											*
10	Steel liners of mills											*
11	Rubber liners and parts of mills											*
12	Abrasion parts of pumps											*
13	Filter's cloth and cleaning cloth											*
14	Wire strips											*
15	Cotton strips											*
16	Plastic sectors											*

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17	Lifter bar, impact bar											*
18	Impact bar											*
19	Shell plate											*
20	Wooden cartons,boxes&pallets											*
21	Great bar											*
22	Steel Pallet											*

A: Mining waste
B: Oily waste
C: Organic Waste
D: Putrescible organic materials
E: High volume and low-risk waste
F: Miscellaneous waste

Analysis of the data shows that, of the 22 types of waste identified at Golgohar Mining and Industrial Complex, six were hazardous because they possessed at least one dangerous UNEP property. These were rock and soil tailings, dry tailings, wet tailings, used oil and grease and used activated carbon. Another 14 showed no hazardous characteristics and were considered to be non-hazardous waste. Despite the small number of types of waste classified as environmentally hazardous, the percentage of each was higher than the percentage of non-hazardous waste because of their high volume. This means that of the 24,461,753 tons of industrial waste produced at in 2015, 24,452,753 tons were environmentally hazardous and 9,000 tons were non-hazardous according to UNEP standards. In other words, more than 99% of the industrial waste in the study area was considered to be hazardous and less than 1% was considered to be non-hazardous (*Figure 2*).Note that of the 24,452,753 tons of hazardous waste, about 19,768,236 tons were rock and soil tailings, which were considered to be high volume and low-risk waste according to the UNEP classification (*Figure 3*).

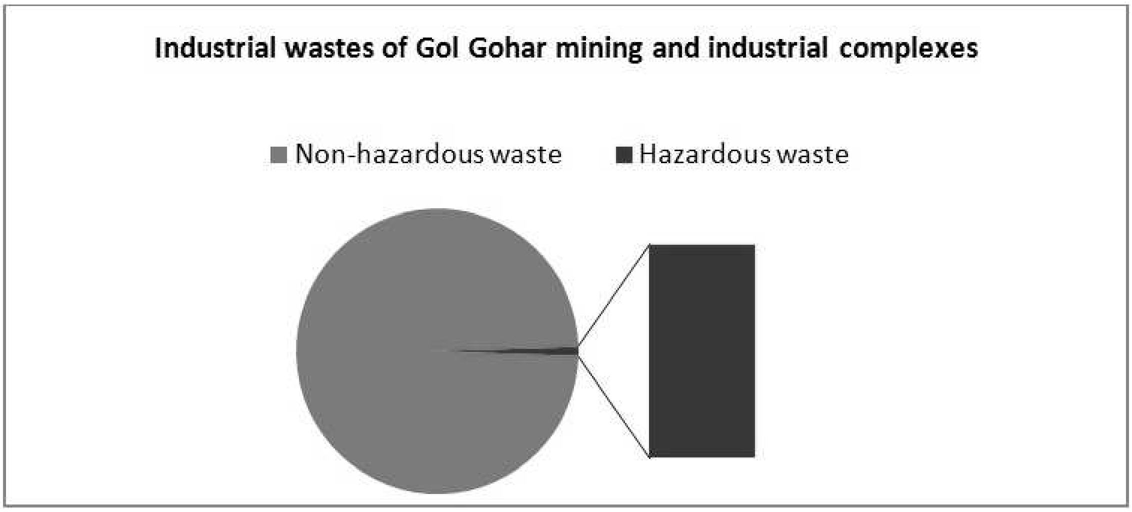

Figure 2. *Percentage hazardous and non-hazardous waste in GolGohar Mining and Industrial Complex according to UNEP's classification in 2015*

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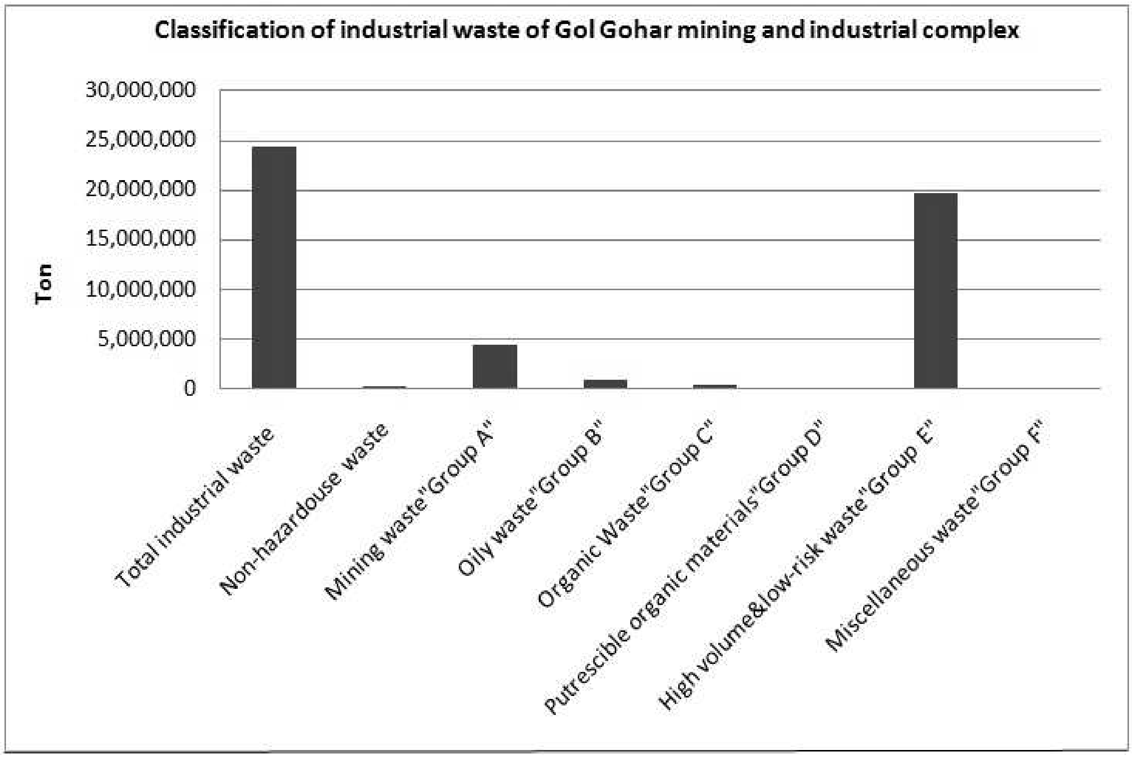

Figure 3. *Classification of all industrial wastes in GolGohar Mining and Industrial Complex according to the UNEP's classification in 2015*

It should be noted that from the total of 24,452,753 tons of hazardous waste, about 19,768,236 tons included tailing rock and soil, which were considered as High volume and low-risk waste according to UNEP's classification (*Figure 3*).

Discussion

Soil and water are important environmental resources which can be contaminated and seriously threatened by improper disposal of industrial waste. It has been suggested that water and soil be protected as a serious investment with the implementation of waste management. Unfortunately, despite the appropriate legal bases in Iran such as the Third and Fourth Development Plans and other environmental laws, gaps and shortcomings in the implementation of industrial waste management has been observed. Practical suggestions for improving the execution of industrial waste management in Golgohar Mining and Industrial Complex have been suggested by the analysis and the results of previous studies in this region.

Reduce waste at the source of production

A basic principle of waste management is that waste reduction should begin at the origin and continue through all stages of production. It is evident that reducing waste at the source is fundamental and more efficient than at other stages. In many cases, the effective use of resources, a decrease in the consumption of raw materials, recycling, and the reuse of waste are accompanied by significant social and economic benefits, such as reduced cost of production and decreased pollution and disposal costs.

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One way of reducing the amount of industrial waste is to change the process. In some cases, manufacturing may contain defects which create waste. Such waste can be reduced by the removal of the defects and modification of the process. A change in the process may be done by modifying equipment, process automation and operation planning (such as regulation of temperature, pressure and amount of raw materials). For example, the volume of tailings in most cases can be reduced when lines and equipment are controlled in order to ensure optimal performance. A defect at any stage of manufacturing, such as crushing, magnetic separation, flotation, filtering, screening and handling can be controlled by continuous control of the lines to reduce tailing production. Rock and soil tailings, however, including waste containing iron (at a lower grade than currently economically viable due to lack of access to advanced technology) and other elements which have economic value, such as copper and vanadium, need further investigation to ascertain their potential use. The volume and amount of waste, together with its danger and toxicity can be reduced through the practice of tailings.

Recycle waste

In the waste management hierarchy, after waste reduction at the source, follows recycling and reuse of waste. It may be possible to use waste again in the same process after recycling at the production unit or could be given to other units for recycling and then used for final processes. If a unit can recycle its waste, the volume of the waste produced will decrease and savings will increase in disposal operations and, most importantly, in their collection and transportation. This is an important step toward protecting the environment.

Because industrial waste is produced in separated form and in high volumes in the manufacturing and industrial units, its recycling become easier and more efficient than municipal solid waste. In the study area, a certain percentage of waste is recycled; thus, it was recommended that recycling of waste be followed with thorough assessment of existing waste production and determination of the potential of industrial waste recycling. Dust captured by the electrostatic precipitator and wet scrubber sludge are taken back into the production process, recyclable metal parts are collected and sold, and used oil is collected and transferred after filtering and treatment. It is evident that all non-hazardous waste, except activated alumina and molecularsieves, are recyclable and can be sold to recycling plants for plastic, rubber, metal and paper. In addition to compensating for part of the purchasing expense of these materials, this can help prepare the raw materials for another industrial unit. This addresses many of the problems and costs associated with landfills and disposal of these materials, which is the responsibility of the manufacturer.

The last alternative for waste recycling is their use for thermal energy. At present, much waste is burned in cement and asphalt kilns or consumed as auxiliary fuel in a fossil fuel furnace. Used rubber and tires and used oil and grease are used in large industrial complexes after output standards have been strongly observed. Used vehicle tires (trucks and lorries) and used oil and grease are common in the study area; their volume means they can be sold to cement and asphalt kilns. This will decrease the volume of waste and also compensate for part of production expenses. Energy recovery of waste is preferred to its disposal.

The chemical composition of the tailings shows that they contain elements such as silica oxide, aluminum oxide, calcium oxide, magnesium oxide, titanium oxide and iron oxide that can be considered for making cement blocks, bricks, ceramics and glass.

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Rock and soil tailings can be used as road infrastructure and in mine rehabilitation programs after harvest and final discharge (*Table 4*).

Table 4. *Chemical characteristics of tailing*

	Silica Oxide SiO_2 (%)	Aluminium Oxide Al_2O_3(%)	Calcium Oxide (CaO) (%)	Magnesium Oxide (MgO) (%)	Titanium Oxide TiO2(%)	Iron Oxide FeO(%)	Iron Fe (%)
Dry tailings	32.48	7.75	6.26	12.53	0.48	3.42	18.24
Wet tailings	29.52	6.34	7.43	15.40	0.34	3.87	36.66

The use of advanced technologies for further concentration can be added to the agenda. Because iron ore concentrationis done by using its magnetic property, iron extraction can be increased by increasing the strength of the magnetic separators. A survey has shown that processing plants in the Golgohar complex can extract 68% of the iron from ore. Increasing the magnetic strength of the separators can allow extraction of a higher percentage of iron. This would reduce the volume of waste and increase production at processing plants, making it more economical.

Because mining tailings contain precious and rare metals in low grades, extraction of these metals can be arranged. In addition to the economic benefits, this can lead to the removal of the risks of heavy metals, which primarily threaten the quality of water and soil and, secondarily, the health of the community.

Mining tailings are potentially suitable for a variety of uses. The aggregation and physical properties of iron mine tailings advocate their use as a primary material in the manufacture of cement and concrete. Mine tailing recycling for use in other industries saves energy and resources and has economic benefits. It also reduces the volume of accumulated mining waste and can reduce problems associated with the accumulation and disposal of mining tailings.

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EFFECTS OF SOME ECOLOGICAL FACTORS ON DISTRIBUTION OF *PRANGOS ULOPTERA* AND *PRANGOS PABULARIA* IN RANGELANDS OF ARDABIL PROVINCE, IRAN

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Abstract. The aim of this study is to assess the effects of some environmental factors on the distribution of *Prangos uloptera* DC. and *Prangos pabularia* Lindl. In the rangelands of Ardabil province, in the north-west of Iran. Six sites with the distribution of the genus *Prangos* (presence) and six sites without *Prangos* (absence) were identified. Three 100m transects were established. On each transect, ten 4m^2 plots were located, and the total canopy cover and density of plants were recorded. Soil samples were collected from a depth of 0–30cm on each transect. Elevation, slope and aspect were extracted from the Digital Elevation Model (DEM), and rainfall, temperature were also extracted from the derived gradient equation and DEM for each plot. Soil parameters such as organic matter (OM), nitrogen (N), phosphate (P), potassium (K), pH, electric conductivity (EC) and texture were determined at the Soil Lab. Normality of data was tested. To compare the related parameters for the sites with the presence and absence of species, the one-way ANOVA was conducted. Cluster analysis was used to classify the collected samples. To determine the variables that were significant in the distinction of each groups and mean comparison, ANOVA and Tukey tests were conducted. To determine the importance of ecological factors for the presence and absence of the selected species, discriminant analysis was conducted. The results of the analysis of variance between presence and absence of *P. uloptera* and *P. pabularia* in habitats demonstrated significant differences for the parameters of elevation, slope, aspect, temperature, EC, pH, OM, nitrogen, phosphorus, clay, silt and sand and total canopy cover, $P<0.01$. The result of grouping sampling plots using cluster analysis showed that plots in three groups were separated by significant differences ($P<0.05$) in the multivariate analysis of variance. The results showed that *P. uloptera* was adapted in habitats with OM of 1.402%, N 0.126%, P 2.627$_{ppm}$, K 258$_{ppm}$, 1992m a.s.l., with average slope of 83.40%, clay 10.90%, silt 37.90%, sand 51.20%, and average precipitation of 353mm. However, *P. pabularia* was adapted in habitats with OM of 0.915%, N 0.088%, P 2.339$_{ppm}$, K 236$_{ppm}$, 1672m a.s.l., and with average slope of 84.80%, clay 13.60%, silt 29.70%, sand 56.70% and average precipitation of 370mm. With regard to the results from discriminant analysis, the percentages of slope, clay, sand, silt, OM, P and pH, aspect, precipitation and temperature play an effective role in the separation and distribution of the species studied. These results can be used to suggest suitable species in management and restoration of rangelands with the same ecological characteristics.
Keywords: *ecological factors, species dispersion, cluster analysis, discriminant analysis*

Introduction

Environmental factors affect the establishment and distribution of plant species (Ashcroft et al., 2011). Identification of factors that affect the distributions of species is an important unresolved issue in ecology (Araújo and Guisan, 2006). Often, there are many combinations of predictors that can explain distributions equally well, especially when environmental factors are correlated, and this introduces uncertainty into the effect of each factor (Platts et al., 2008; Murray and Conner, 2009). Therefore, the first step in rangeland management is to determine the habitats of plants and the effective factors affecting their distribution. Autecological field or laboratory studies have long

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been relied upon to reveal environmental variables that impede a plant's migration into surrounding territory (Arundel, 2005). These variables—or limiters—shape various spatial aspects of individual plant species' distributions. Knowing specific influences on plants is important in many fields, but particularly for climate reconstruction based on fossil plant remains (Arundel, 2002). The presence and distribution of plant species in rangeland ecosystems are not random, but such factors as climate, soil, topography, anthropological and others play major roles in their development (Akbarlou and Nodehi, 2016). Determination of the factors that control the presence and distribution of rangeland species is one of the main objectives in the rangeland ecosystems studies.

Though relationships between plant and both soil properties and other environmental factors have been well developed for some plants, comparable understanding of how a variety of plant species in native rangelands responds to soil properties and other environmental factors is poorly developed (Chuangye et al., 2015; Ghorbani et al., 2015; Sahragard and Zare Chahouki, 2015). Xu et al. (2008a) have studied the relationships between vegetation, soil and topography in the dry valley of China. Their results confirmed that plant diversity was mainly correlated with soil water content, and soil water content was mainly determined by soil texture, especially clay content. Yibing (2008), by conducting the Principal Component Analysis (PCA) and Canonical Correlation Analysis in China, showed that physical and chemical features of the soil, including humidity, salinity and acidity affected homogeneity of plant communities on the regional scale. Zhang and Dong (2010), in the study of relationships between environmental factors and vegetation diversity in Lesi plateau of China, reported that elevation, soil type, slope and aspect were important factors in Lesi zones' recovery, and had determinant roles in vegetation distribution. Zare et al. (2011), in a study on the relationship between environmental factors and plant distribution in arid and semiarid areas, reported that soil texture, salinity, effective soil depth, available N, K, OM, lime and soil moisture, were the major soil factors responsible for variations in the pattern of vegetation. Ghorbani and Asghari (2014), in the study of ecological factors affecting the distribution of *Festuca ovina* in the southeastern rangelands of Sabalan, reported that *F. ovina* is more compatible with higher altitudes and lower temperatures and does not tolerate soil salinity, and is more compatible with a pH of 7.1 to 7.3. OM, P and K provide better conditions for its growth.

To improve the management of rangelands and to offer a baseline for restoration attempts, an understanding of the factors that determine the rangelands vegetation distribution and composition is required. For this purpose, this study was conducted to identify the roles of topography, climate and soil components, in the distribution of *Prangos uloptera* and *P. pabularia,* in the rangelands of Ardabil province in north-western Iran. Identification of these parameters in a given ecosystem helps one apply appropriate management for restoration and development in the present and in similar rangelands.

Materials and Methods

Study area

The study area was selected in the rangelands of Ardabil province, Iran (37° 12' to 38° 07' N and 47° 51' to 48° 48' E) (*Figure 1*). Six habitats were selected with the genus *Prangos* distribution. *P. uloptera* were distributed in four habitats and *P. pabularia* were in two habitats. According to meteorological stations in the region (with a 10-year

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period—2006 to 2015), the mean precipitation is 360mm/year. Also, maximum and minimum precipitations respectively occur in February and July. The mean of annual temperature is 9℃, the average annual minimum temperature is 3.2℃ and the average annual maximum temperature is 14.8℃.

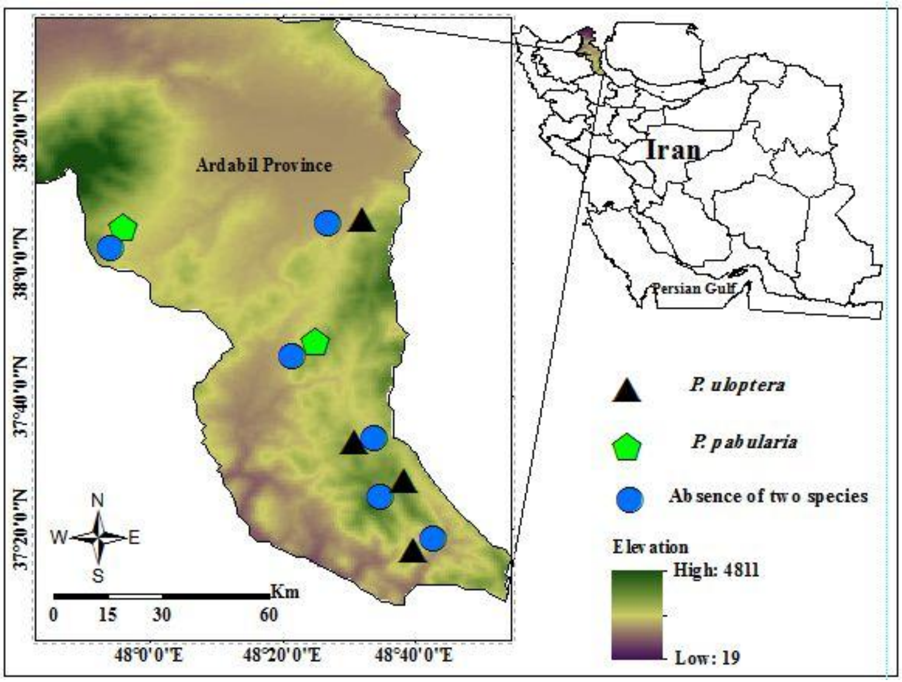

Figure 1. *Location of the study area and sampling locations for presence and absence of the selected species in Ardabil province and Iran*

Sampling method

Initially, in order to recognize the distribution of the genus *Prangos*, an overall study was conducted by literature review (Rechinger, 1987; Mozaffarian, 2007; Azimi et al., 2011; Teimoorzadeh et al., 2015; Ghorbani et al., 2015). Moreover, fieldworks were conducted in Ardabil province to select the habitat of the genus *Prangos*. Finally, six habitats were identified with *P. uloptera* and *P. pabularia* distributions. In each habitat, initially one site with genus *Prangos* distribution was selected as the presence of the selected species. Near to each of the selected sites, another site with the absence of genus *Prangos* were selected in the same habitat (overall, for the presence and absences of genus *Prangos*, 12 sites were selected). A Digital Elevation Model (DEM) map was derived using 1:25000 topographic maps of the National Cartographic Center of Iran, with 10 m horizontal and vertical accuracy. Slope and aspect maps were derived from the DEM for the selected habitats and sites. By considering the studies conducted on the rangelands of Ardabil province (i.e. Ghorbani and Asghari, 2014; Ghorbani et al., 2015), in each site three 100 m transects were established. The first one was selected randomly, and the second one as parallel with 500 m distance, and the third one perpendicular to both. By considering the abovementioned literature and using minimal area method (Kent and Coker, 1996), the size of plots was determined as $4m^2$. The number of plots was also determined by considering the literature (i.e. Ghorbani and Asghari, 2014; Ghorbani et al., 2015), and the pre-samples taken were calculated

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(Kerbs, 1999) as about 10 samples. Overall, ten $4m^2$ plots were selected for each transect, wherein the distance between the plots (from the centre of each one) was 10m, for the 12 selected sites, for the presence and absence of species. Within each plot (presence and absence of the species), total canopy cover, percentage and density of plants, including the selected species, were recorded. In each site, nine soil samples were collected from the depth of (0–30 cm) (Northup et al., 1996). The soil properties—such as N, OM, EC, pH, P, K and texture—were measured via laboratory analysis at the Soil Lab of Ardabil University.

Data analysis

Kolmogorov-Smirnov test was used to assess the normality of data. To analyse significant difference between the effects of environmental factors on the presence or absence of two selected species, the one-way ANOVA was used. For grouping the collected samples on the basis of the recorded ecological factors, cluster analysis was used. To determine the variables that assume significance in the distinction of identified groups and to compare these, ANOVA and Tukey test were used. The importance of each measured variable in the distribution of selected species was analysed using discriminant analysis. The ArcGIS10 was used to extract the base map and SPSS18 software was used for data analysis.

Results

The results of the analysis of variance between presence and absence of *P. uloptera* and *P. pabularia* in habitats demonstrated significant difference for the parameters of elevation, slope, aspect, temperature, EC, pH, OM, nitrogen, phosphorus, clay, silt and sand and total canopy cover, $P<0.01$ (*Table 1*). The clustering of sampling plots, using cluster analysis, indicated that the plots are divided into three groups ($P<0.01$) in the multivariate analysis of variance (*Figure 2*). The discrimination based on the plots revealed that Group 1 is related to the absence of the two species in the habitats, Group 2 shows the habitat of *P. uloptera*, and Group 3 indicates the habitat of *P. pabularia*. The results of multivariate analysis of variance among the groups proved that the resultant groups had significant differences in all the variables, except for the precipitation level and potassium, $P<0.01$ (*Table 2*). The elevation, organic matter and silt in the habitat of *P. uloptera* (Group 2) were higher than the habitat of *P. pabularia* and the habitats without the study species, which had significant difference, $P<0.01$ (*Table 2*). In the habitat of *P. pabularia* (Group 3), the percentage of sand and temperature were higher than other habitats, which had significant difference ($P<0.01$), as well as the habitats without the study species had the highest electrical conductivity, clay and phosphorus compared to other habitats ($P<0.01$). The slope in the habitats of *P. uloptera* and *P. pabularia* had the highest percentage (over 83%) and the habitats without the study species had lower percentage (mean slope of 66%). The results showed that *P. uloptera* was adapted in habitats with average OM of 1.402%, N 0.126%, P 2.627_{ppm}, K 258_{ppm}, 1992m elevation a.s.l., slope 83.40%, clay 10.90%, silt 37.90%, sand 51.20%, and also average precipitation of 353mm. However, *P. pabularia* was adapted in habitats with average OM 0.915%, N 0.088%, P 2.339_{ppm}, K 236_{ppm}, 1672m elevation a.s.l., average slope 84.80%, clay 13.60%, silt 29.70%, sand 56.70%, and also average precipitation of 370 mm.

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Table 1. *Analysis of variance of environmental factors and their effects on the presence or absence of P. uloptera and P. pabularia*

		Sum of Squares	df	F
Elevation (m)	Between Groups	36923549.214	2	19.033**
	Within Groups	39461.356	33	
Slope%	Between Groups	64745.081	2	125.221**
	Within Groups	2881.912	33	
Aspect	Between Groups	500.211	2	9.949**
	Within Groups	27.389	33	
Precipitation (mm)	Between Groups	1370579.678	2	1.384ns
	Within Groups	4879.101	33	
Temperature (C^o)	Between Groups	403.843	2	12.331**
	Within Groups	27.899	33	
EC (Ds/m)	Between Groups	1.336	2	31.840**
	Within Groups	0.242	33	
pH	Between Groups	8.403	2	34.046**
	Within Groups	1.603	33	
OM%	Between Groups	99.024	2	17.665**
	Within Groups	9.800	33	
N%	Between Groups	0.864	2	11.705**
	Within Groups	0.057	33	
P (ppm)	Between Groups	370.154	2	6.722**
	Within Groups	13.940	33	
K (ppm)	Between Groups	1770318.244	2	1.976ns
	Within Groups	19596.654	33	
Clay%	Between Groups	10490.046	2	83.939**
	Within Groups	421.744	33	
Silt%	Between Groups	29395.534	2	17.834**
	Within Groups	279.344	33	
Sand%	Between Groups	21747.143	2	34.008**
	Within Groups	1668.023	33	
Density *P. uloptera*	Between Groups	35.600	2	197.225**
	Within Groups	3.220	33	
Density *P. pabularia*	Between Groups	2.722	2	277.272**
	Within Groups	0.752	33	
Total Canopy Cover	Between Groups	6430.225	2	101.987**
	Within Groups	245.368	33	

**, ns: Respectively significant at 1% and non-significant

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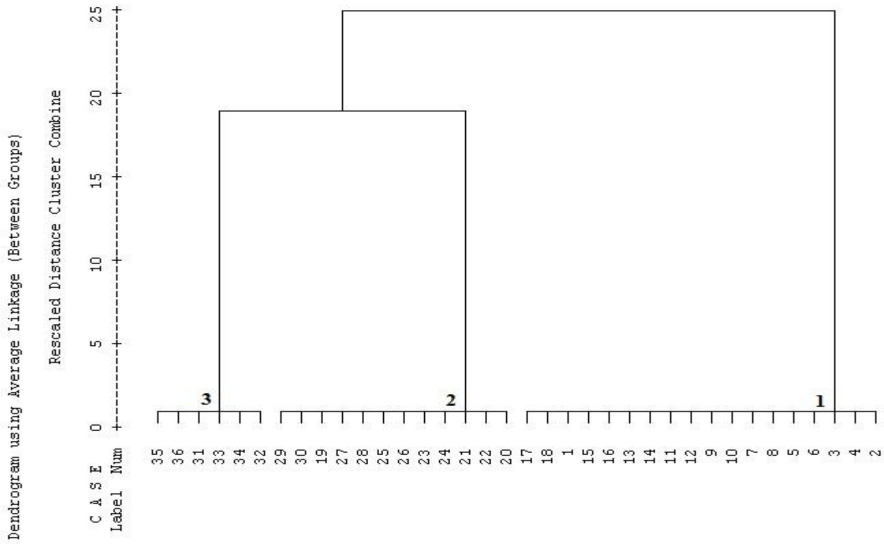

Figure 2. *Cluster analysis of ecological factors and density of P. uloptera and P. pabularia using cluster analysis method*

Table 2. *Comparison of measured characteristics in the conditions of the presence and absence of species*

	Absence of two species (1)		***P. uloptera* (2)**		***P. pabularia* (3)**	
	Mean	Std. Error	Mean	Std. Error	Mean	Std. Error
Elevation (m)	1861^{b}	28.000	1992^{a}	26.000	1672^{c}	22.000
Slope%	66.300^{b}	0.919	83.400^{a}	0.765	84.800^{a}	1.084
Aspect	0.012^{b}	0.001	0.013^{b}	0.001	0.012^{b}	0.001
Precipitation (mm)	359^{a}	4.620	353^{a}	6.843	370^{a}	1.539
Temperature (C^{o})	8.943^{b}	0.081	8.665^{b}	0.061	9.499^{a}	0.196
EC (Ds/m)	0.537^{a}	0.005	0.487^{b}	0.004	0.480^{b}	0.008
pH	7.679^{a}	0.011	7.535^{b}	0.014	7.666^{a}	0.021
OM%	1.181^{b}	0.037	1.402^{a}	0.055	0.915^{c}	0.058
N%	0.113^{a}	0.004	0.126^{a}	0.005	0.088^{b}	0.006
P (ppm)	2.875^{a}	0.086	2.628^{ab}	0.092	2.339^{b}	0.051
K (ppm)	250^{a}	3.188	258^{a}	9.810	236^{a}	4.403
Clay%	17.800^{a}	2.387	10.900^{c}	1.399	13.600^{b}	2.305
Silt%	34.600^{b}	2.560	37.900^{a}	2.737	29.700^{c}	2.606
Sand%	47.600^{c}	3.439	51.200^{b}	3.675	56.700^{a}	3.400
Density of *P. uloptera*	0.000^{b}	0.000	0.667^{a}	0.017	0.000^{b}	0.000
Density of *P. pabularia*	0.000^{b}	0.000	0.000^{b}	0.000	0.240^{a}	0.022
Total Canopy Cover%	46.150^{c}	0.389	56.200^{a}	0.957	52.450^{b}	0.657

Same letters in a row indicate no significant difference.

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By using the discriminant analysis of habitats based on environmental factors and the results obtained, the two functions justified respectively 69.8% and 32% and a total of 100% of the variance of total data. Moreover, the canonical correlation coefficient showed that the Functions 1 and 2 were able to discriminate well between the groups (*Table 3*). *Table 4* indicates the values of Wilks' lambda for functions, which increase from the Function 1 through the Function 2. The index closer to zero shows more appropriate estimated function in discrimination of groups, so Functions 1 and 2 had proper estimation in discrimination of groups. Considering the significance of chi-square values at the level of <0.01, the mean groups are different. In each of the Functions 1 and 2, the study parameters had different coefficients, so that the factors influencing the grouping of habitats as well as the distribution of the species studied can be determined, thanks to these coefficients (*Table 5*). Accordingly, the slope, clay, sand, silt, pH, organic matter, temperature, aspect, potassium and precipitation are effective in discrimination of habitats and the distribution of the species studied. Clay and temperature, having the highest standardized coefficients, as well as potassium and organic matter, having the lowest standardized coefficients, respectively had the maximum and minimum impacts on the first detection function. The average annual precipitation has the greatest effect on Function 2. Owing to structural coefficients (*Table 5*), slope, clay, EC, potassium and sand in the first function, as well as pH, silt, organic matter, elevation, temperature, nitrogen, aspect, potassium and precipitation in the second function, show the most correlation with functions formed. According to stepwise discriminant analysis, the functional equation can be set as Function 1 using canonical discriminant function coefficients, where factors involving pH, organic matter, potassium, slope, aspect, clay, silt, sand, phosphorus, and average temperature have been included into the equation.

$$Y_1 = -1.437\ pH - 0.365\ OM - 0.002\ K + 0.041\ Slope - 0.010\ Aspect - 0.320\ Clay - 0.068\ Silt + 0.121\ Sand + 0.016\ P_{(mm)} + 1.184\ T_{(C^{\circ})} - 4.988 \quad \text{(Eq.1)}$$

The results of the classification of habitats studied by discriminant analysis are shown in *Table 6*. The percentages presented in this table show the matching level of observed and predicted cases. If the two species are in sites without Function 1 function properly, they determine membership of the Group 1 in 100 per cent of cases. If the data of absence of two species are in the first function, the function will properly determine membership of the Group 1 in 100 per cent of cases. If the data of *P. uloptera* are in the Function 1, the function will properly determine membership of the Group 2 in 99.20 per cent of cases. If the data of *P. pabularia* are in Function 1, the function will properly determine membership of Group 3 in 98.30 per cent of cases. Overall, 99.40% of the main grouped cases have been properly classified. Accordingly, the results of this investigation indicated the effects of environmental factors on discrimination of habitats of studied species and the sites without the two species (*Figure 2*). In this figure, Group 1 represents the sites without the two species, Group 2 reflects the habitats of *P. uloptera*, and Group 3 shows the habitats of *P. pabularia*.

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Table 3. *Eigenvalues and the percentage of variance explained by the two first functions*

Function	Eigenvalue	Variance (%)	Cumulative (%)	Canonical correlation
1	7.062[a]	69.8	69.8	**0.936**
2	3.048[a]	30.2	100.0	**0.868**

a. First 2 canonical discriminant functions were used in the analysis.

Table 4. *Wilks' lambda values in discriminant analysis*

Test of function(s)	Wilks' lambda	Chi-square	df	Sig.
1 through 2	0.031	1225.138	24	**0.000**
2	0.247	491.507	11	**0.000**

Table 5. *Standardized canonical discriminant function coefficients and structure matrix in the sites studied*

	Standardized canonical discriminant function coefficients		**Structure matrix**	
	Function		Function	
	1	**2**	**1**	**2**
Slope%	0.428	.0510	**0.315***	-0.027
EC (Ds/m)	-	-	**-0.259***	0.060
Clay%	**-1.458**	0.129	**-0.237***	0.154
P (ppm)	-	-	**-0.148***	-0.022
Sand%	0.895	0.060	**0.143***	0.124
pH	-0.221	0.182	-0.111	**0.184***
Silt%	-0.595	-0.205	0.001	**-0.181***
OM%	-0.192	-0.380	0.008	**-0.180***
Elevation (m)	-	-	0.005	**-0.171***
Temperature (C°)	**1.259**	0.209	0.011	**0.150***
N%	-	-	0.037	**-0.149***
Aspect	-0.789	-0.109	0.057	**-0.103***
K (ppm)	-0.174	0.310	-0.002	**-0.060***
Precipitation (mm)	0.980	**0.521**	0.004	**0.050***

Table 6. *The results of classification using discriminant analysis*

Classification results[a]					
	site	Predicted group membership			Total
		Absence of two species	*P. uloptera*	*P. pabularia*	
Original %	**Absence of two species**	100.0	0.0	0.0	100.0
	P. uloptera	0.8	99.2	0.0	100.0
	P. pabularia	0.0	1.7	98.3	100.0

a.99.4% of original grouped cases correctly classified.

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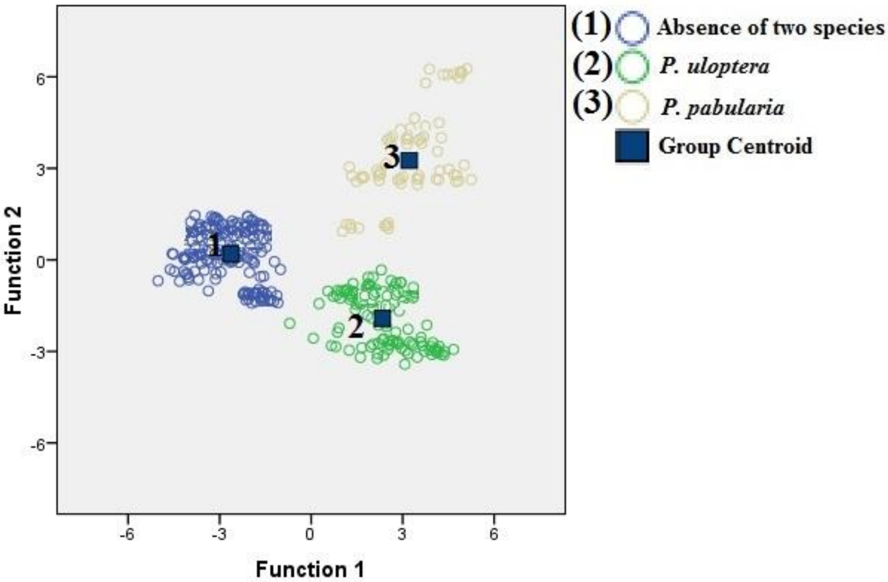

Figure 3. *Canonical discriminant functions in the conditions of presence and absence of species*

Discussion and conclusion

Establishment of plant communities occurs under the terms of climate and environmental conditions of the habitat; the plants are not randomly distributed across the globe. It is possible distribution of any plant in specific geographic limitation because any plant has specific environmental requirements that, if have in a certain area of growth and reproduction, these requirements must be provided. Comparison of the selected ecological factors in the habitats with the presence or absence of species demonstrated that most of the variables had significant differences. According to the field studies, no *P. pabularia* was found in sites with elevations >1,700 metres and *P. uloptera* had greater density in the higher elevations. Thus, the ecological requirements of these species appear to be different, and environmental characteristics of the distribution ranges of these species will have different effects. According to Ghorbani et al. (2015), the most important factors in the separation of plant species were reported to be elevation, slope percentage, soil texture, and depth in the southeastern rangelands of Sabalan. Moreover, Zhang and Dong (2010), in their study of the relationships between environmental factors and vegetation diversity, observed that elevation, soil type, slope and aspect were important factors in Lesi zones' recovery and played determinant roles in vegetation distribution. The precipitation and temperature were the study factors which, in turn, are affected by the elevation, and had significant differences among habitats in the study—so that *P. pabularia* in sites with higher precipitation and temperature and *P. uloptera* in sites with lower precipitation and temperature are more compatible. Ghorbani and Asghari (2014) also emphasized that precipitation and temperature can affect the distribution of *Festuca ovina* in southeastern rangelands of Sabalan, Ardabil, Iran. The organic matter content was the factor that had a significant impact on the distribution of the species studied. The organic matter content in the habitats of *P. uloptera* was more than in the habitats of *P. pabularia*. The density and total canopy cover were higher in the habitats of *P. uloptera* than the habitats of *P. pabularia*, probably due to the large amount of litter in these habitats. Soil organic matter within the rangeland system provides more nutrients for plant growth, which

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results in a positive feedback, as more plant biomass is likely to produce more soil organic matter (Ryals et al., 2014). Additionally, organic matter affects soil chemicals, physical and biological properties, and this has been suggested as the single most important indicator of soil quality (Xu et al., 2008b; Ryals et al., 2014; Mirzaei Mossivand and Bahrami, 2015).

The results of many studies showed that OM was one of the soil characteristics affecting the distribution of vegetation that was in agreement with the results of Salama et al. (2013), Zare et al. (2011), He et al. (2007) and Abd El-Ghani and Amer (2003). Kooch et al. (2007), in their study of ecological distribution of indicator species and effective soil factors in Mazandaran province, showed that the distribution of vegetation was correlated with soil properties such as soil texture, P, OM, N and pH. According to the results, both *P. uloptera* and *P. pabularia* species prefer often soils with sandy-loam textures, and this means that they are more compatible with lighter textured soils. The soil texture affects the penetration and retention of water, and the availability of water and nutrients in plants (Sperry and Hacke, 2002). Soil texture controls dynamics of soil organic matter in many simulation models or organic matter decomposition and formation (Raich et al., 1991), and influences infiltration and moisture retention and the availability of water and nutrients by plants (Sperry and Hacke, 2002). Some studies, such as of Xu et al. (2008a) and Zarei et al. (2010), proved that the soil texture (clay, silt and sand) is one of the most important factors affecting plant type distribution. Results of Abbadi and El Sheikh (2002) and Davies et al. (2006) showed that soil texture is the most important factor in the separation of ecological groups. The results of discriminant analysis showed that slope, clay, sand, silt, pH, organic matter, temperature, aspect, potassium and precipitation contribute to the discrimination of the habitats and the distribution of the species studied. Ghorbani et al. (2015) and Jafarian et al. (2010) also stated that the use of discriminant analysis in the relationship between environmental factors and distribution of vegetation has been beneficial. As the findings were presented, if the eigenvalues and canonical correlation coefficient are higher, the function obtained will be stronger, and the accuracy of classification will be greater. Discrimination variables that entered into the functions belonged to all the climatic, soil and topographic factors, representing the right choice of the parameters. In general, it can be stated that the functions resulting from discriminant analysis could be applied to the same species in other sites, using the findings of the present study, and identifying factors influencing the presence and absence of the species studied in the rangelands of Ardabil, thereby saving time required for similar studies. Details of this study can be useful in the optimal management of the rangelands. In addition, according to the survey results and discrimination of the habitats related to the species studied and the effects of ecological factors on their distribution, these findings could be used in the improvement and restoration of the same rangelands.

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SENSITIVITY ANALYSIS IN INVESTMENT PROJECT OF BIOGAS PLANT

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Abstract. The article shows the practical value of biogas as the second generation biofuel. All the projects dealing with biogas are subjected to external risks, such as the change of market condition, customer needs, governmental regulation, etc. In conditions of uncertainty it is necessary for administration to concentrate on decision-making. Fluctuations of sales volume, energy resources and raw material prices, etc. should be taken into account. Sensitivity analysis can predict the result of negative external phenomena. We developed the economic-mathematical model for the analysis of biogas complexes sensitivity. The profitability index as a criterion for the effectiveness of investment projects is a special feature in this model. The calculations show that biogas optimal distribution provides much larger gross income. We also suggested the methodology for sensitivity analysis implementation in investment projects for biogas complex creation. According to our calculations, the most stable project has utilization both biomethane (as motor fuel) and carbon dioxide. We demonstrate that the use of the profitability index as a criterion for an investment project gives higher critical values of input external factors, that endows assured profitability of an investment project.
Keywords: *biomethane, profitability index, net present value, critical point, carbon dioxide, motor fuel*

Introduction

Exhaustible fossil energy resources and ecological problems are among the major threats of modern civilization. Therefore, nowadays there are acute problems such as efficient use of traditional energy resources, implementation of energy-saving technologies, reduction of energy production intensity and the use of alternative energy resources including renewable ones (Brower et el., 2006; Stern, 2007).

Science has developed and used the civilizational approach to the understanding of the history of mankind. The transition from one stage of civilization maturity to another takes place due to deep qualitative changes of productive forces of the society and growth of labor productivity. These changes are impossible without an increase in the use of energy resources.

In the researchers' opinion, the power engineering is developed as follows (*Fig. 1.*):

- reducing energy intensity of production;
- increased use of fossil energy resources, which reserves exceed the reserves of petroleum;

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- the use of renewable energy sources;
- the use of energy resources has to comply with envirinmental standards.

Since the end of the twentieth century, there has been a tendency to reduce the use of hydrocarbon fossil fuels due to intensive utilization of renewable energy, including biofuels. Therefore, there has been a spiral trend in the use of energy at a higher technological level. In ancient times, biofuels were used in the primary form (wood or agricultural waste), but nowadays they are mainly utilized in a more convenient, processed form such as briquettes, liquid or gaseous biofuels.

The first generation biofuels are produced from crops. As a result, it decreases food producing. More than one billion people are starving regularly worldwide. In 2008 this fact forced the United Nations to appeal to suspend production of biofuels from crops which can be used for food (LSB, 2013). Thus, production of the second-generation biofuels (from non-food plant resources) is a promising direction. It will not increase the deficit of food crops (Patyka et al., 2016). Animal and crop waste, municipal and industrial waste water, solid waste landfill are substrates for biogas production. Such gaseous biofuel can be used as a substitute for natural gas and motor fuels. Therefore, biogas production is an encouraging step forward.

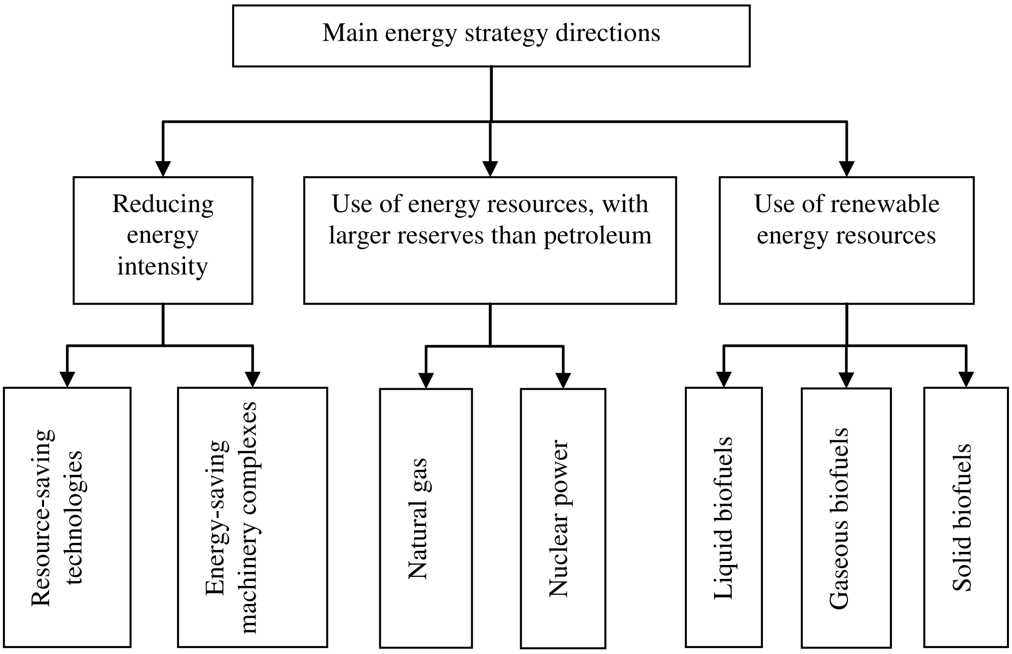

Figure 1. *Main energy strategy directions in power engineering*

A significant number of countries are increasing biogas production (ePURE, 2014; Alberici and Toop, 2013). Thus, in China, biogas production has reached 15 billion m^3 per year. This equals about 7,5 million tons of oil equivalent. Futhermore, in the EU, biogas production exceeds 13 million tonnes of oil equivalent (EBA, 2013).

Moreover, biogas meets recent environmental requirements. In accordance with the requirements of the Directive 2009/28/EC, reduction of greenhouse gas emissions in the implementation of renewable energy technologies should be at least 35% compared to fossil fuels use. Biogas use reduces greenhouse gas emissions by 56-86%, depending on

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the substrate and way of its utilization. This renewable gaseous fuel meets the requirements of the second generation biofuels (Geletukha at al., 2014). Thus, biogas production and use can be an attractive investment. Its use allows both to meet the requirements of the post-industrial society and make a significant contribution to the energy security.

All investment projects, including building of biogas plants, are subjected to risks. They are forced with uncertainty. It is a result of a lack of firm data concerning conditions and parameters of investment project implementation. The administration should focus on making decisions to achieve maximum profitability in the condition of uncertainty. Therefore, it is necessary to assess the impact of negative external factors on effectiveness of an investment project (Pikler, 2014).

Evaluation of investment projects is possible while applying different methods, such as the break-even analysis, sensitivity analysis, scenario method, theory of games, decision-making theory, etc. One of the simplest and most effective methods is sensitivity analysis. This analysis is a calculating procedure used to predict the impact of input data on output results of a project. This procedure is often used for investment decision-making related to investment project evaluation in conditions of uncertainty (Adler, 1987; Kalinichenko et al., 2016). However, the methodology of sensitivity analysis for investment projects of biogas producing is still not developed enough and needs to be investigated.

The aims of this study were to clarify the procedural technique for the sensitivity analysis of a biogas plant investment project, determine the degree of input values set influence on their cost-effectiveness and reveal the difference in results for the accepted criteria: *NPV* and *PI*.

Literature Review

One of the challenges for the biofuel industry, including biogas plants, is the high level of uncertainty. These uncertainties complicate the assessment of investment decisions (Kim et al., 2011). The input variables are regarded as uncertain. These are the investment costs, discount rate, sale prices, sales volume, economic plant life, etc. The sales volume consists of electricity, heat, fertilizer, biomethane, by-product (carbon dioxide). They are every year changeable and dependent from the current market prices. Both the achievable sales volume and the selling price are uncertain (Blohm, 2006; Hoffmeister, 2008; Pikler, 2014).

The sensitivity analysis is suitable if there is no statistical data. It allows us to identify factors on which managers’ attention should be focused during project implementation (Willem and Groenendaal, 1998; Borgonovo and Peccati, 2004). The purpose of the sensitivity analysis is selecting “critical” variables of the model which have the biggest influence on the criteria of an investment project and cause the most significant changes in these parameters.

The theoretical risk classification, assessment methods and risk management have been studied by a number of scientists. The analysis of these works shows that the risk is a complex, multifaceted phenomenon. There are different interpretations of the concept.

The studies were focused both on the general sensitivity analysis of investment projects and the risk in general (Milanović et al., 2010; Burja and Burja, 2009; Analti, 2003; Jovanovic, 1999). The sensitivity analysis is a mandatory attribute of the financial analysis of energy projects.

Federica Cucchiella and Indiano de D'Amado have evaluated financial feasibility of biomethane plants and proposed a mathematical model. The Net Present Value and Discounted Payback Period are the indicators of the above. The sensitivity analysis on the critical variables was also conducted (Cucchiella and D'Amado, 2016). General aspects of the sensitivity analysis were studied by Mirela Iloiu and Diana Csiminga. In their research, viability of investment projects are based on the Internal Rate of Return (*IRR*) and Net Present Value (*NPV*) criteria (Iloiu and Csiminga, 2009).

Despite the number of studies (Menind and Olt, 2009; Kossmann et al., 2009; Pingping, 2010; Arnórsso, 2011), the sensitivity analysis is still not very well understood and this reduces the quality of management decisions. The sensitivity analysis of biogas projects is used to facilitate decision making under uncertainty. This can provide an adequate insight into the possible problems for the decision maker (Mészáros, 2014).

Peter Jovanovic considered the Net Present Value, Internal Rate of Return and Payback Period as criteria of investment projects (Jovanovic, 1999). In the authors' previous study, only *NPV* was accepted as a criterion (Gavrish and Perebijnos, 2015). The *NPV* is an absolute criterion, which cannot give an unambiguous answer about a project's suitability. In our opinion, the Profitability Index (*PI*) is a more appropriate criterion. That is why both *NPV* and *PI* criteria are used for this study.

Research Methodology

The methods used in this study include on-site data collection and literature review. The on-site data collection includes technical information about biogas plants accumulating, their efficiency, risk and uncertainty. The literature review helps to do a financial analysis.

First of all, we defined a set of criteria as a basis for the investment project evaluation. The assessment of an investment project is actually based on an estimation of future cash-flows. We also selected and identified a set of input key factors.

The resulting calculations are the values of individual criteria determining the values of certain input variables to determine the maximum (or minimum) values that certain variables can take if the investment project remains profitable. Finally, the results were analyzed and interpreted. It helps us to prevent or remove adverse impacts and make certain improvements.

Mathematical Model Development

In this study, the net present value and profitability index were chosen as criteria of the investment project. The *NPV* and *PI* have been selected as they are the main parameters for investors while making decisions.

The *NPV* is a difference between the present value of cash inflows and present value of cash outflows. The profitability index is a ratio of payoff to investment of a suggested project. It allows to quantify an amount of value created per unit of investment. If the *PI* is more than 1.2, a project is acceptable.

The sensitivity analysis procedure is as follows:

- identify all the variables used to calculate the output of economic analyses;
- carry out a qualitative analysis for an impact of the variables in order to select those having little or marginal elasticity;
- having chosen the significant variables, it is possible to evaluate their elasticity;

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- identify the critical variables, apply the chosen criterion.

In the investment project evaluation, there is a set of input values (income, costs, discount rate, value of investments, etc.) and a set of output ones (*NPV* and *PI*). Having used the input values, it is possible to determine certain individual output values (*Fig. 2*) (Stone, 1988).

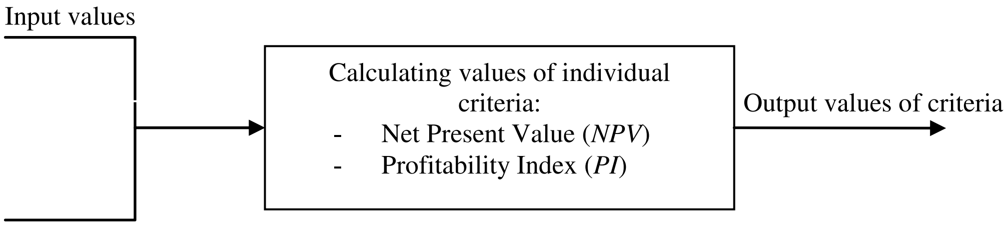

Figure 2. *Calculation of individual criteria using input and output values*

Let us analyze the critical points, i.e. determine the level of project-specific factors leading to the zero net present value. With respect to the biogas plant, the *NPV* is defined by the following formula

$$NPV = \sum_{i=1}^{n} \frac{GI_i - OC_i}{(1+d)^i} - \left(I_0 + \sum_j \frac{I_{aj}}{(1+d)^{T_j}} \right), \qquad \text{(Eq. 1)}$$

where GI_i is a gross revenue from the biogas plant operation (use of electrical energy, thermal energy, biomethane as motor fuel, carbon dioxide, manure, etc.) in the i^{th} time period, EUR; OC_i is operating expenses in the ith period, EUR; d is the discount rate; n is the economic plant life, years; I_0 is the initial cost of the total investment, EUR; I_{aj} is ~~a~~ the cost of the j^{th} additional investments, EUR; T_j is time of the j^{th} additional investment, years.

While projects are planned to operate and be efficient for a time period of approximately 20 - 25 years, we assume that their plant life is 20 years (Gkamplia et al., 2012; Henning, 2011). But a depreciation period of some equipment (pumps, stirrers, heating system, combined heat and power generation, etc.) is shorter and is about 7 - 10 years (Henning, 2011). So, additional investments are made to renovate some equipment with shorter lifetimes.

As for the profitability index, its value should not be less than 1.2

$$PI = \frac{\sum_{i=1}^{n} \frac{GI_i - OC_i}{(1+d)^i}}{\left(I_0 + \sum_j \frac{I_{aj}}{(1+d)^{T_j}} \right)} \geq 1.2 \qquad \text{(Eq. 2)}$$

Next, we will consider three ways of biogas utilization with:

- combined heat and power generation;
- motor fuel replacement;

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- motor fuel substitution and carbon dioxide use (resulting in biogas upgrading).

We propose to study an impact of the following major risk factors:

- the initial investment value;
- the additional investments value;
- the substrate cost;
- the biogas plant load factor;
- the discount rate;
- the actual biogas plant life;
- biogas plant operating expenses;
- revenue from electric power sale;
- revenue from heat sale;
- revenue from biogas (biomethane) sale (as motor fuel);
- revenue from carbon dioxide (by-product of biogas upgrading) sale.

The factors having a low variation are the most risky elements of the investment project. The relative deviation of each factor is determined as follows:

$$\Delta F = \frac{F_{cr} - F_o}{F_o} \cdot 100\% \qquad \text{(Eq. 3)}$$

where F_o is the influence factor value established according to the initial prognosis; F_{cr} is the critical influence factor value.

To determine the degree of influence of each factor on the net present value, we should to determine the elasticity coefficient. Therefore, we use the following formulas:

$$E_{NPV/F} = \frac{dNPV / NPV}{dF / F} \qquad \text{(Eq. 4)}$$

or

$$E_{PI/F} = \frac{dPI / PI}{dF / F} \qquad \text{(Eq. 5)}$$

These expressions determine the elasticity at a particular point. In many cases (including dependence of the linear model), elasticity is different at various points. Thus, it is advisable to calculate the average value of the elasticity coefficient as a ratio of percentage changes in *NPV*, *PI* and *F*

$$E_{NPV/F} = \frac{\Delta NPV / \overline{NPV}}{\Delta F / \overline{F}} \qquad \text{(Eq. 6)}$$

or

$$E_{NPV/F} = \frac{\Delta PI / \overline{PI}}{\Delta F / \overline{F}} \qquad \text{(Eq. 7)}$$

Having taken into account that (for *NPV*)

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$$\Delta NPV = NPV(F_{cr}) - NPV(F_o),$$
$$\Delta F = F_o - F_{cr},$$
$$\overline{F} = 0{,}5 \cdot (F_o + F_{cr}),$$
$$\overline{NPV} = 0{,}5 \cdot (NPV(F_o) + NPV(F_{cr})),$$

we get the final expression for the elasticity coefficient (for *NPV*)

$$E_{NPV/F} = \frac{(NPV(F_{cr}) - NPV(F_o))/(NPV(F_o) + NPV(F_{cr}))}{(F_{cr} - F_o)/(F_o + F_{cr})} \quad \text{(Eq. 8)}$$

A positive value of the elasticity coefficient indicates the coincidence of trends of both factors and the net present value, and a negative one – that the directions of the changes of a factor and *NPV* have different signs. In equation (1) at the critical point $NPV(F_{cr}) = 0$. If $NPV(F_{cr}) = 0$, then the elasticity factor is

$$E_{NPV/F} = \frac{(0 - NPV(F_o))/(NPV(F_o) + 0)}{(F_{cr} - F_o)/(F_o + F_{cr})} = -\frac{F_o + F_{cr}}{F_{cr} - F_o} \quad \text{(Eq. 9)}$$

Similarly, we obtain the expression for the profitability index

$$E_{PI/F} = \frac{(PI(F_{cr}) - PI(F_o))/(PI(F_o) + PI(F_{cr}))}{(F_{cr} - F_o)/(F_o + F_{cr})} \quad \text{(Eq. 10)}$$

In equation (2) at the critical point $PI(Fcr) = 1.2$.

Sensitivity Analysis Procedure

Performing the sensitivity analysis procedure involves the study of changes in the indicator, considering the remaining constants. If we take into consideration that the additional capital investments are made once in a biogas plant lifetime, the equation (1) is simplified and takes the following form

$$NPV = \sum_{i=1}^{n} \frac{GI_i - OC_i}{(1+d)^i} - \left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}} \right) \quad \text{(Eq. 11)}$$

If we assume that there is a regular cash flow, the annual return will be

$$R_0 = GI_i - OC_i = const \quad \text{(Eq. 12)}$$

So,

$$NPV = \sum_{i=1}^{n} \frac{GI_i - OC_i}{(1+d)^i} - \left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}} \right) = R \cdot \sum_{i=1}^{n} \frac{1}{(1+d)^i} - \left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}} \right) \quad \text{(Eq. 13)}$$

Let us determine the critical point, i.e. the value of specific factors leading to zero net

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present value.

All the factors influencing the project effectiveness can be divided into two groups: the first group includes general ones (independent on technological features of the investment project) and the second group (dependent on technology). The values of the initial investment, additional capital investment, lifetime, discount rate belong to the first group. The values of the biogas plant load factor, revenue from energy resourses (biomethane, electric and heat power) and carbon dioxide sale belong to the second group.

The actual investment costs may deviate from the established ones according to the initial prognosis. They may be a result of tax legislation amendments, for example. The critical value of the initial investment is

$$I_{cr} = R_0 \cdot \sum_{i=1}^{n} \frac{1}{(1+d)^i} - \frac{I_{aj}}{(1+d)^{T_j}} \quad \text{(Eq. 14)}$$

where R_0 is the annual return according to the initial prognosis.

Such substitution can be made in case of constant difference in the gross income and operation expenditure during the project lifetime.

The critical value of additional investment is

$$I_{ajcr} = (1+d)^{T_j} \cdot \left(R_0 \cdot \sum_{i=1}^{n} \frac{1}{(1+d)^i} - I_0 \right) \quad \text{(Eq. 15)}$$

The critical value of the annual return is

$$R_{cr} = \frac{\left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}} \right)}{\sum_{i=1}^{n} \frac{1}{(1+d)^i}} \quad \text{(Eq. 16)}$$

The critical value of the project lifetime can be determined as the discounted payback period (provided it is shorter than the additional investment period). If we take into account that

$$\sum_{i=1}^{n} \frac{1}{(1+d)^i} = \frac{1-(1+d)^{-n}}{d} \quad \text{(Eq. 17)}$$

the discounted payback period is equal to

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$$Tcr = DPP = -\frac{\ln\left(1 - d \cdot \frac{I_0 + \frac{I_{aj}}{(1+d)^{Tj}}}{R_0}\right)}{\ln(1+d)} \quad \text{(Eq. 18)}$$

The critical value of the discount rate is found from the equation

$$0 = \sum_{i=1}^{n} \frac{R_0}{(1+d_{cr})^i} - \left(I_0 + \frac{I_{aj}}{(1+d_{cr})^{T_j}}\right) \quad \text{(Eq. 19)}$$

where d_{cr} is the critical value of the discount rate.

The root of the equation (19) is found numerically. In fact, the critical value of the discount rate is equal to the internal rate of return, i.e. $d_{cr} = IRR$.

Let us consider the factors that are directly determined by the parameters of the biogas plant and its products. A gross income from the biogas plant operation and all related costs are determined as follows. Based on this, we consider the impact of the following factors on the project effectiveness: the substrate cost, operating expenses, heat and electricity use, biomethane use to replace conventional motor fuel (gasoline or diesel), carbon dioxide use.

The substrate cost is equal to

$$SC = M_s \cdot S_{pr} \quad \text{(Eq. 20)}$$

where M_s is the annual consumption of the substrate, ton; S_{pr} is the substrate price (production cost), EUR/t.

The critical value of the substrate price (production cost) can be found from the following formula

$$R_0 - R_{cr} = Ms \cdot \left(S_{pr_0} + \left(S_{pr_{cr}} - S_{pr_0}\right)\right) \quad \text{(Eq. 21)}$$

where S_{pr_0} is the substrate price (production cost) in the base case, EUR/t; $S_{pr_{cr}}$ is the critical value of the substrate price (production cost), EUR/t.

So, the critical value of the substrate price (production cost) is

$$S_{pr_{cr}} = \frac{R_0 - R_{cr}}{M_s} = \frac{R_0 - \frac{\left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}}\right)}{\sum_{i=1}^{n} \frac{1}{(1+a)^i}}}{M_s} \quad \text{(Eq. 22)}$$

The critical value of operating expenses is

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$$OC_{cr} = GI - \frac{\left(I_0 + \frac{I_{aj}}{(1+d)^{T_j}}\right)}{\sum_{i=1}^{n} \frac{1}{(1+d)^i}} \quad \text{(Eq. 23)}$$

Let us consider three possible options of the biogas plant project. A company (a founder of the biogas plant project) may consume both heat and electric energy. Three options of biogas utilization have been considered:

- the company's energy requirements are covered with heat and electric energy produced by biogas plants;
- upgraded biogas (biomethane) is used as motor fuel (instead of petroleum fuels), carbon dioxide (a by-product of upgrading) is not used;
- both upgraded biogas (biomethane) and carbon dioxide are utilized or sold at the market price.

Biomethane and carbon dioxide are primary meant to be sold externally.

The gross income for the first option is determined as follows:

$$GI = W_e \cdot E_{pr} + Q_e \cdot H_{pr} + M_m \cdot M_{pr} \quad \text{(Eq. 24)}$$

where W_e is electric energy production and use, kWh; Q_e is heat energy production and use, kWh; E_{pr} is the electric energy price, EUR/kWh; H_{pr} is the heat price, EUR/kWh; M_m is the annual manure production, t; M_{pr} is the manure price, EUR/t.

So, the critical values of the electricity and heat consumption will be

$$W_{e\,cr} = W_e - \frac{R_o - R_{cr}}{E_{pr}} \quad \text{(Eq. 25)}$$

and

$$Q_{e\,cr} = Q_e - \frac{R_o - R_{cr}}{H_{pr}} \quad \text{(Eq. 26)}$$

The critical value of the manure utilization volume is

$$Mm_{cr} = Mm_o - \frac{R_o - R_{cr}}{M_{pr}} \quad \text{(Eq. 27)}$$

The equation for determining the gross income in the second option is

$$GI = V_b \cdot \frac{Q_b}{Q_f} \cdot F_{pr} + Ms \cdot S_{pr} \quad \text{(Eq. 28)}$$

where F_{pr} is the motor fuel price, EUR/liter; V_b is annual biogas production according to the initial prognosis, m^3; Q_b is lower heat values of biogas, MJ/m^3; Q_f is lower heat values of motor fuel, MJ/kg.

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The critical value of biogas volume (in the form of biomethane) to substitute petroleum motor fuels is

$$V_{b\,cr} = V_{b0} - \frac{R_0 - R_{cr}}{F_{pr}} \cdot \frac{Q_f}{Q_b} \qquad \text{(Eq. 29)}$$

The equation to determine the gross income in the third option is

$$GI = V_b \cdot \frac{Q_b}{Q_f} \cdot F_{pr} + 0{,}01 \cdot \xi \cdot V_b \cdot CD_{pr} + M_m \cdot M_{pr} \qquad \text{(Eq. 30)}$$

where V_{b0} is annual production of biogas according to the initial prognosis, m^3; ξ is carbon dioxide content in biogas,%; CD_{pr} is the carbon dioxide price, EUR/l.

The critical value of biogas volume (in the form of biomethane) to substitute petroleum motor fuels is determined by the formula (30), and the volume of carbon dioxide sale is

$$V_{cd\,cr} = 0{,}01 \cdot \xi \cdot Vb_{cr} = 0{,}01 \cdot \xi \cdot V_{b0} - \frac{R_0 - R_{cr}}{CD_{pr}} \qquad \text{(Eq. 31)}$$

If the maximum possible change of a factor does not result in reduction of the net present value to zero, the critical value of the net present value is

$$NPV^* = NPV(F^*) \qquad \text{(Eq. 32)}$$

where F^* is the maximum possible value of a factor.

Let us consider the determining of critical points if profitability index is used as a criterion of an investment project. At the same time, we are going to take into account the fact that the profitability index values should be at least 1.2.

The critical value of the initial investment can be found from the equation of the profitability index

$$PI^* = 1{,}2 = \frac{\sum_{i=1}^{n} \frac{R_0}{(1+d)^i}}{\left(I_0 + \frac{I_{aj}}{(1+d_{cr})^{T_j}} \right)} \qquad \text{(Eq. 33)}$$

So,

$$I_{cr} = \frac{\sum_{i=1}^{n} \frac{R_0}{(1+d)^i}}{PI^*} - \frac{I_{aj}}{(1+d_{cr})^{T_j}} \qquad \text{(Eq. 34)}$$

The critical value of the additional investment is

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$$I_{ajcr} = (1+d_{cr})^{T_j} \cdot \left(\frac{\sum_{i=1}^{n} \frac{R_0}{(1+d)^i}}{PI^*} - Io \right) \quad \text{(Eq. 35)}$$

The critical value of the annual return is

$$R_{cr} = PI^* \cdot \frac{\left(Io + \frac{I_{aj}}{(1+d_{cr})^{T_j}} \right)}{\sum_{i=1}^{n} \frac{1}{(1+d)^i}} \quad \text{(Eq. 36)}$$

The critical value of the economic plant life is to equal to the Discounted Payback Period (*DPP*)

$$T_{cr} = DPP = -\frac{\ln\left(1 - PI^* \cdot d \cdot \frac{I_0 + \frac{I_{aj}}{(1+d)^{Tj}}}{R_0} \right)}{\ln(1+d)} \quad \text{(Eq. 37)}$$

Example of Sensitivity Analysis

In order to exemplify the sensitivity analysis, the below biogas plant project is used. The annual capacity of biogas plant is equal to 599,1 thousand m^3. The initial data for different variants is listed in *Table 1*.

Table 1. *Initial data for biogas plant project options*

№	Parameters	Unit	Options		
			1	2	3
1	Initial investment	thousand EUR	765.22	884.78	913.48
2	Additional investment	thousand EUR	191.30	310.87	339.57
3	Period of additional investment	year	11.00		
4	Annual biogas production	thousand m^3	590.21		
5	Lifetime of project	year	20.00		
6	Discount rate		7.50		
7	Annual consumption of electricity by a biogas plant	thousand kWh	92.97		
8	Annual consumption of thermal energy by a biogas plant	thousand kWh	449.36		
9	Annual use of biogas to	thousand m^3	0.00	282.73	282.73

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	substitute diesel fuel				
10	Annual carbon dioxide production	thousand m^3	0.00	0.00	171.75
11	Company's annual electrical power consumption	thousand kWh	1146.96	0.00	0.00
12	Company's annual thermal energy consumption	thousand kWh	1029.86	0.00	0.00

To assess financial risks, we will use the above factors. The resulting calculations are demonstrated in T*able 2*.

Table 2. *Project sensitivity analysis*

№	Parameters	Relative deviation					
		Option 1		Option 2		Option 3	
		Critical change of a factor	Elasticity coefficient	Critical change of a factor	Elasticity coefficient	Critical change of a factor	Elasticity coefficient
1	Initial investment	0.61	-4.30	0.36	-6.55	1.58	-2.26
2	Additional investment	5.37	-1.37	2.27	-1.88	9.44	-1.21
3	Plant's economic life	-0.59	2.37	-0.52	2.88	-0.77	1.58
4	Discount rate	1.13	-2.77	0.84	-3.39	2.57	-1.78
5	Biogas plant-load factor	-0.35	4.67	-0.24	7.43	-0.58	2.47
6	Substrate cost value	1.45	-2.38	1.00	-3.00	4.53	-1.44
7	Operating expenses	0.76	-3.64	0.15	-14.62	0.58	-4.45
8	Electric energy use	-0.41	3.91	–	–	–	–
9	Thermal energy use	-0.99	1.03	–	–	–	–
10	Manure sale*	-1.00	0.90	-0.73	1.76	-1.00	0.54
11	Diesel fuel substitute	–	–	-0.12	15.51	-0.55	2.64
12	Carbon dioxide sale	–	–	–	–	-0.94	1.13

* *Zero scope of manure use does not lead to a decrease in the net present value to zero in the first and third options.*

The obtained data shows the main risk factors for the project:

- the first option – biogas is utilized to generate electric or/and heat power;
- the second and third options —biogas is used to substitute diesel fuel.

The projects' options prove to be stable to the value of an additional investment and substrate cost value. Use of the *PI* as a criterion for an investment project gives lower values of the input critical variables (*Table 3*).

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***Table 3.** Comprehensive assessment of the project sensitivity*

№	Parameters	Option 1		Option 2		Option 3	
		Critical change of a factor when using NPV	Critical change of a factor when using PI	Critical change of a factor when using NPV	Critical change of a factor when using PI	Critical change of a factor when using NPV	Critical change of a factor when using PI
1	Initial investment value	0.61	0.32	0.36	0.11	1.58	1.12
2	Additional investment value	5.37	2.86	2.27	0.67	9.44	6.68
3	Project lifetime	-0.59	-0.38	-0.52	-0.17	-0.77	-0.66
4	Biogas plant load factor	-0.35	-0.22	-0.24	-0.08	-0.58	-0.49

The sensitivity analysis of the project was carried out graphically. It is proposed to improve the classic method by introducing a line of the minimum allowable value project performance criteria (*NPV* = 0 or *PI* = 1.2). In percentage terms, the *NPV* = -100%, while for the *PI* it is

$$\delta PI = \frac{PI^* - PI}{PI} \cdot 100\% \quad \text{(Eq. 34)}$$

Fig. 3 shows that 20% decrease of return and 30% increase of investment cause critical reduction of *PI*. In the second option, 10% deviation of investment or return makes the project unprofitable (*Fig. 4*). The third option proves to be the most stable one (*Fig. 5*). The sensitivity analysis shows that the *NPV* and *PI* approximately respond to changes in the biogas plant capacity.

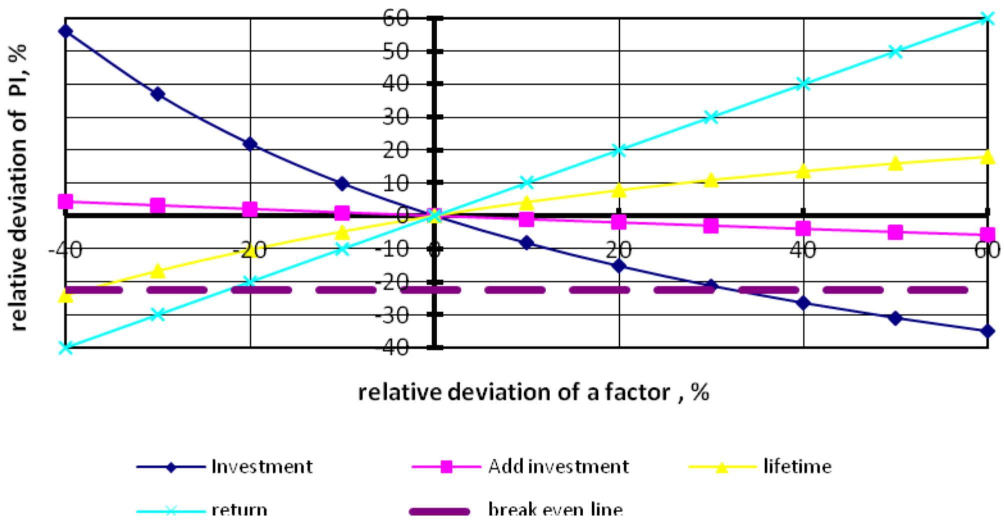

***Figure 3.** Sensitivity analysis of the first option*

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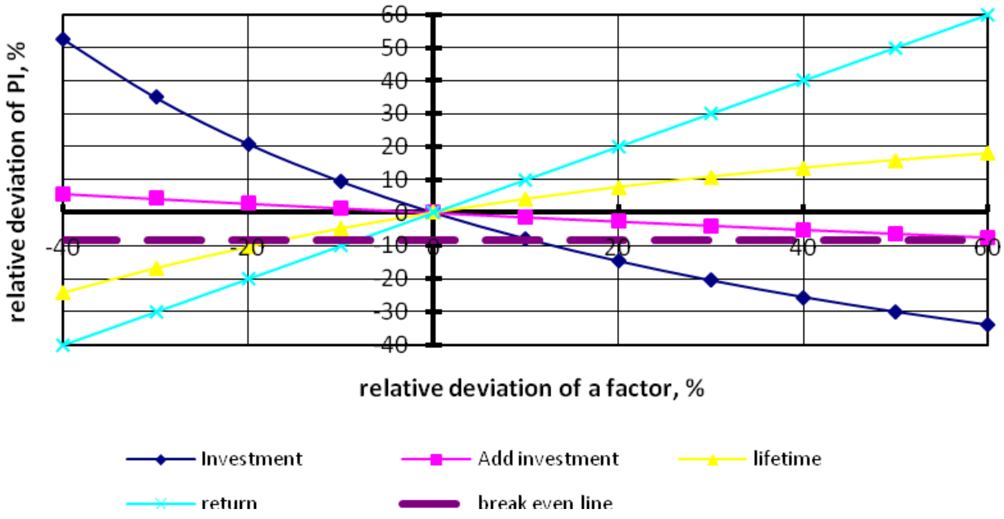

Figure 4. *Sensitivity analysis of the second option*

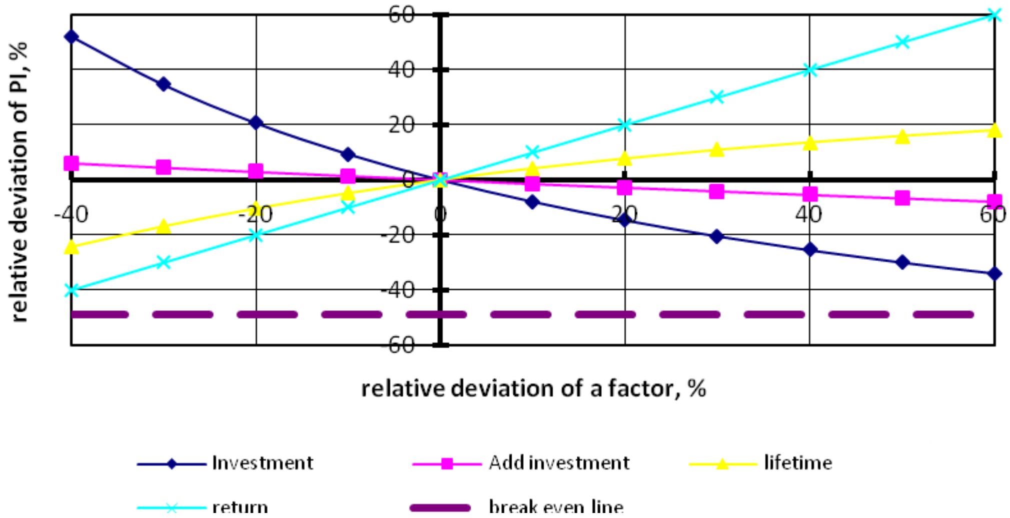

Figure 5. *Sensitivity analysis of the third option*

Conclusions

The profitability index is proposed to be used as a criterion of an investment project for the sensitivity analysis procedure. It can guarantee profitability of an investment project at the critical points. The economic and mathematical model for sensitivity analysis of an investment project of biogas plant has been developed. It takes into account the main input factors having influence on a project criterion.

According to the calculations for the biogas plant projects, the most risky option is to use biogas as vehicle fuel and not to use its by-product (carbon dioxide). The most sustainable project is using both biomethane (as vehicle fuel) and carbon dioxide for sale. This study has revealed that biogas plant progects are more sensitive to biogas

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plant load factor, and less sensitive to the additional investment and substrate cost.

For further research, we suggest taking into account the scenario method to study probability of external factors. The sensitivity analysis methodology can be improved. The price ratio of biomethane and petroleum fuels should be subject to future study. Moreover, location and capacity have a high level of uncertainty.

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PREDICTED DEVELOPMENT OF THE CITY OF NITRA IN SOUTHWESTERN SLOVAKIA BASED ON LAND COVER-LAND USE CHANGES AND SOCIO-ECONOMIC CONDITIONS

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Abstract. The aim of this paper is to predict possible future development of the city of Nitra, southwestern Slovakia. We proposed three scenarios of possible further development of the city (negative, progressive and realistic) based on mapping and evaluation of land cover and land use changes in Nitra from 1949 to 2015. Also taking into account the socio-economic development of the city with an emphasis on the current state and recent changes. The SWOT analysis in three domains (social, economic, environmental) was prepared and used for scenario development as well. The spreading of a built-up area is a natural consequence of city development. It is connected with processes of urbanization and industrialization that are accompanied with loss of agricultural land. Changes caused by these processes are clearly visible in Nitra and also within its vicinity. The structure of land use development has varied over the years. At the beginning of the period studied, changes connected with agriculture production dominated, while presently, the changes that prevail are connected with urbanization and industrialization.
Keywords: *CORINE, future development prediction, scenario approach, SWOT analysis*

Introduction

All the important driving forces of landscape changes are related to the population growth and the lifestyle becoming increasingly more urban and more mobile (Antrop, 2005). Spreading of the urban area accompanied with urban population growth and economic development is a global trend. Urbanization causes land cover and land use changes that are usually related to economic growth. As a country moves from a rural-agricultural base to an urban-industrial base, urbanization and economic development go hand in hand (Davis and Henderson, 2003). The pace and magnitude of landscape changes depends upon increasingly faster technological innovations and societal changes (Antrop, 2000). Therefore, the urban landscape is a result of mutually connected economic, demographic, socio-cultural, political, technological and environmental processes that are shaping its character (Ira, 2011). One of the consequences of urbanization is soil sealing, which is a change from agricultural, forest or other semi-natural or natural landscape into an urban and industrial landscape. This includes areas covered with buildings and infrastructure, urban greenery or sport and leisure facilities (EEA, 2015).

Understanding of landscape changes requires a sound understanding of the underlying processes that can be triggered by different driving forces (Hersperger and Bürgi, 2009). Papers focused on land cover changes and their driving forces in Central

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and Eastern Europe were published by e. g. Skokanová et al. (2012), Jarský and Pulkrab (2013), Lieskovský et al. (2013), Hartvigsen (2014), Kanianska et al. (2014), Munteanu et al. (2014) Smiraglia et al. (2015), Lima et al. (2016), Opršal et al. (2016) and Skokanová et al. (2016).

The structure of a cultural landscape is largely determined by human decisions – by direct or indirect impacts of human activities. In Europe, the human impact is mainly determined by agriculture but differs from region to region (Mander and Jongman, 1998). Nitra is situated in a major agricultural area of the Slovak Republic. That is why the agricultural land is most affected by changes in the study area. Changes in cultural and agricultural land in Slovakia were mapped by e. g. Oťahel et al. (2012), Ivanová (2013), Tarasovičová et al. (2013), Lieskovský et al. (2014) and Kopecká et al. (2015).

The study area includes urban areas and also agricultural and forest areas. Because of that, the landscape structure of the study area is considerably diversified and types of changes are quite numerous. Land cover and land use in Nitra were mapped by various authors, e. g. Bugár et al. (2006), Mišovičová (2008), Hreško et al. (2015) and Haladová and Petrovič (2015).

The aim of this paper is to propose scenarios of further possible development of the city based on mapping and evaluating of the land cover and land use changes in Nitra and two surrounding villages from 1949 to 2015, taking into account the socio-economic development of the city with an emphasis on the current state.

Materials and Methods

Study area

The study area consists of the cadastral areas of Nitra and the villages Lužianky and Ivanka pri Nitre. The size of the studied area is 12 304.73 ha.

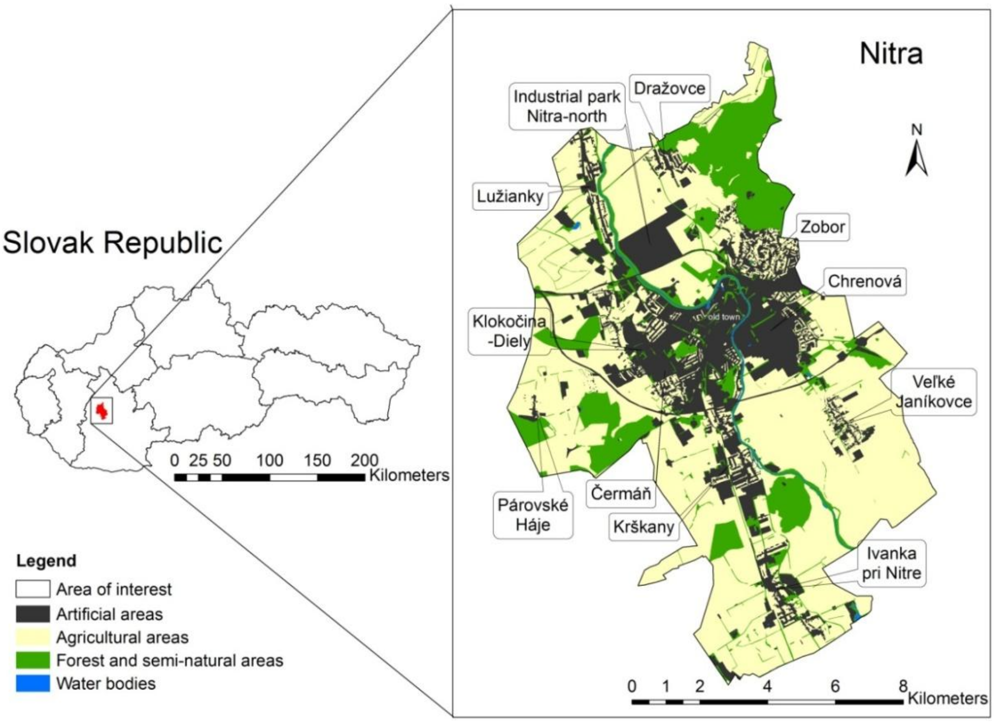

Figure 1. *Location of the study area within the Slovak Republic*

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Nitra is the capital of Nitra county and Nitra district. It is situated in the south-western part of Slovakia, on the border of the Tríbeč mountain range and the Podunajská lowland (*Fig. 1*). This location creates specific conditions suitable for the development of agriculture, industry and forestry. Agriculture has a long tradition in this region. However, it has declined over the past few years, especially viniculture and fruit growing mainly due to the urbanization and industrialization. Thanks to the R1 expressway, the development of industry is supported by good traffic connections, especially with the western and central areas of Slovakia.

Methods

We evaluated the size of land cover classes in five years (1949, 1974, 1990, 2003 and 2015) and changes to their size between these years.

We used the map layers of land cover for the years 1949, 1990 and 2003 created for the publication Landscape of Nitra and its surroundings (Hreško et al., 2006). However, we changed classification legend of landscape classes in these layers, to unify the legend used in all layers. This new classification was created by Petrovič et al. (2009).

Then we created map layers of land cover for the years 1974 and 2015. We prepared the map layer for the year 1974 by analysis of aerial photographs provided by the Topographic institute of the Slovak Republic; the layer for the year 2015 by analysis of aerial photographs made in 2010 (provided by EUROSENSE Slovakia and GEODIS Slovakia), free satellite images (Google maps, Bing maps, Mapy.cz) and field survey.

For classification, we used the first and third levels of legend to the CORINE land cover methodology (Heymann et al., 1994; Bossard et al., 2000). The third level distinguishes 44 classes, in the first level, this methodology has five basic classes:

- Artificial areas
- Agricultural areas
- Forest and semi-natural areas
- Wetlands
- Water bodies

For spatial data processing and creating of map layers we used ArcGIS 10.1 software.

We calculated the area of land cover classes for each year. Then we evaluated changes between the years using multi-temporal analysis, when we merged all data about land cover classes in all studied years into one layer and then we compared their size.

We evaluated land use changes by a six-digit code, where the first three digits represent codes of land cover from one year and the next three digits represent land cover from the next year. Every six-digit code was assigned to the type of change based on the methodology created by Cebecauerová (2007). We added two new types of changes – Forest management (F) and Overgrowing (O) to this classification.

We identified a total of 17 types of changes in the study area:

- Increase of woody vegetation (a) - planting of new forests, planting of alleys and so on
- Increase of woody vegetation, loss of agricultural land (a L)
- Loss of woody vegetation (D) - any loss of wooded areas, such as cutting down a forest, an alley or park vegetation, which is followed by the creation of meadows or grasslands

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- Extensification of agriculture (e) - big and heavily agriculturally used fields being transformed into meadows, abandoned grassy areas or agriculturally used gardens
- Forest management (F) - cutting down a forest to plant a new forest or forest nursery
- Intensification of agriculture (i) - transformation from meadows or small fields (narrow-strip fields) into big, heavily agriculturally used fields
- Intensification of agriculture, loss of woody vegetation (i D)
- Overgrowing (O) - spontaneous growth of shrubs and trees in abandoned areas
- Overgrowing, loss of agricultural land (O L)
- Remediation (R) - process of demolishing built-up areas and their transformation into some kind of vegetation; for example, alley, park, meadow or abandoned area with shrubs and trees
- Industrialization (t) - process of constructing factories, industrial parks, sewage treatment plants or any technical objects.
- Industrialization, loss of woody vegetation (i D)
- Industrialization, loss of agricultural land (t L)
- Urbanization (u) - construction of family houses, construction of blocks of apartments, creation of playgrounds, gardens around family houses and so on
- Urbanization, loss of woody vegetation (u D)
- Urbanization, loss of agricultural land (u L)
- Other changes/Unclassified (oz) - very small changes within the higher level of classification

These types of changes are generalized and one type of change may contain several variations of a specific change.

A lot of areas remained unchanged and were classified as Unchanged area (bz).

We divided landscape elements into five degrees of land use intensity according to Olah et al. (2009). Every group was assigned coefficient from 1 to 5:

1 – Very low land use intensity (tree vegetation, bedrock baring and raw soils, water bodies)
2 – Low land use intensity (shrub vegetation, young forest)
3 – Medium land use intensity (clearings, grass-herbal vegetation, patterns of agriculture land)
4 – High land use intensity (intensively cultivated agricultural land, residential greenery)
5 – Very high land use intensity (built-up and urban areas)

For determination of range and trend of changes, we calculated the intensity of land use and its changes during the studied years according to Olah et al. (2009). We calculated partial changes of land use intensity from the difference between coefficients of landscape elements (Eq. 1). A positive value indicates an increase of land use intensity (intensification) and a negative value indicates a decrease of land use intensity (extensification).

The overall relative change of land use intensity is expressed as the sum of partial changes using the equation:

$$I_R = i_{2-1} + i_{3-2} + \dots i_{m-n} \qquad \text{(Eq. 1)}$$

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where: I_R = relative change of intensity of land use, i_{2-1} = partial intensity of change between the second and first time horizon.

The resulting values may vary in range from -4 to +4

The absolute intensity of change reflects the sum of absolute values of partial changes. Which means, that it disregards their direction (intensification or extensification). The resulting values are therefore always positive. Through the absolute change of land use intensity we are able to identify areas in landscape that were affected by changes the most. We are also able to quantify number of these changes.

We also evaluated socio-economic data that has influenced land cover over the studied years. Socio-economic data was seen as a driving force of land cover changes. As a part of socio-economic analysis, we elaborated SWOT analysis for Nitra, focused on the current state of social, economic and environmental indicators.

We finally created three scenarios of possible city development in the next 20 years based on the acquired information.

Results

Development of land cover and land use intensity in the city of Nitra during the period 1949 to 2015

Agricultural land dominated throughout all of the years studied. However, its size decreased over the whole period. On the other site, artificial areas increased. Forest and semi-natural areas changed their size very slightly during the studied period. Water bodies covered less than 1% of the area in all studied years. Wetlands were located in the area only in 1949. Information about the percentage of land cover classes in each of the studied years is shown in *Table 1*.

Table 1. *Percentage of land cover classes in studied years, first level of classification*

	1949	**1974**	**1990**	**2003**	**2015**
Artificial areas	7,13	13,75	16,74	18,12	23,45
Agricultural areas	71,67	69,61	68,37	63,48	58,89
Forest and semi-natural areas	20,18	16,09	14,27	17,88	17,15
Wetlands	0,15	0,00	0,00	0,00	0,00
Water bodies	0,87	0,55	0,61	0,52	0,51

In the year 1949, 76.61% of the area had medium land use intensity, 10.04% of the area had very low, 7.37% of the area had very high and 5.97% of the study area had high land use intensity.

In the year 1974, 66.39% of the area had high land use intensity, 10.83% of the area had very low, 10.40% of the area had very high, 9.38% of the area had medium and 3.00% of the area had low land use intensity.

In the year 1990, 65.08% of the area had high land use intensity, 13.50% of the area had very high, 12.23% of the area had very low, 8.39% of the area had medium and 0.81% of the area had low land use intensity.

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In the year 2003, 65.06% of the study area had high land use intensity, 14.07% of the area had very high, 11.29% of the area had very low, 5.41% of the area had medium and 4.17% of the study area had low land use intensity.

In the year 2015, 61.09% of the study area had high land use intensity, 18.89% of the area had very high, 10.96% of the area had very low, 4.66% of the area had medium and 4.40% of the area had low land use intensity.

Comparison of land use intensity during the studied years is shown in *Figure 2*.

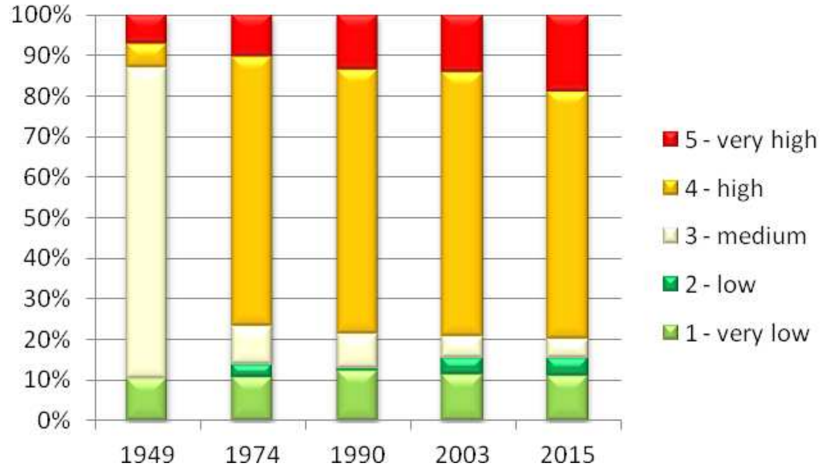

Figure 2. *Intensity of land use during the studied years*

Changes of land cover and land use intensity

Period 1949–1974

Between the years 1949 and 1974, 80.07% of the study area was changed and 19.03% of the area remained unchanged. Information about the extent of all types of changes is in *Table 2*.

Table 2. *Types of changes between the years 1949–1974*

Type of 0change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)
a	1.36	167.44	**i D**	0.26	32.54	**t**	0.34	42.03
a L	2.50	303.40	**O**	0.78	95.83	**t D**	0.01	1.28
bz	19.03	2,341.94	**O L**	0.04	4.61	**t L**	2.46	303.07
D	0.61	75.08	**oz**	1.10	135.27	**u**	0.86	106.39
e	1.25	153.39	**R**	1.35	165.77	**u D**	0.03	3.09
F	0.57	70.63	**R a**	0.05	5.94	**u L**	5.72	703.49
i	60.07	7393.16	**R i**	1.51	185.32	**Z**	0.14	16.95

a - Increase of woody vegetation, **a L** - Increase of woody vegetation, Loss of agricultural land, **D** - Loss of woody vegetation, **e** - Extensification of agriculture, **F** - Forest management, **i** - Intensification of agriculture, **i D** - Intensification of agriculture, Loss of woody vegetation, **O** – Overgrowing, **O L** - Overgrowing, Loss of agricultural land, **R** - Remediation, Recultivation, **t** – Industrialization **i D** – Industrialization, Loss of woody vegetation, **t L** - Industrialization, Loss of agricultural land, **u** – Urbanization, **u D** - Urbanization, Loss of woody vegetation, **u L** - Urbanization, Loss of agricultural land, **oz** - Other changes/Unclassified

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The biggest change was intensification of agriculture (i), which affected 60.07% of the area. On the other hand, extensification of agriculture (e) affected only 1.25% of the area. Changes to agricultural land were connected mainly with collectivization and nationalization of private property. Urbanization on agricultural land (u L) affected 5.72% of the area. A large influence on the urbanization process was the construction of the Chrenová housing development. Remediation of unfit constructions on the border of city centre caused a remediation (R) process that affected 1.35% of the area. Remediation connected with intensification of agriculture (R i) affected 1.51% of the area and industrialization on agricultural land (t L) affected 2.46% of the area. The largest factory built in this period was Plastika. Other changes (oz) and changes smaller than 1.00% of the area affected 3.63% of the study area.

Intensity of land use changed 79.52% of the area. Intensification affected 68.91% of the area and extensification affected 10.60% of the study area.

Period 1974–1990

Between the years 1974 and 1990, 18.35% of the study area was changed and 81.65% of the area remained unchanged. Information about the extent of all types of changes is shown in *Table 3*.

In this period the construction of the Chrenová housing development continued and the construction of two new housing developments - Klokočina and Diely - started. The exhibition center Agrokomplex was also built in this period. This is why urbanization was the most extensive change that affected 2.48% of non-agricultural land (u) and 3.09% of agricultural land (u L). Intensification of agriculture (i) affected 2.70% of the area and in 1.89% of the area, tree and shrub vegetation was cut down for the purpose of creating new agricultural land (i D). On the other hand, extensification of agriculture (e) affected 2.33% of the area. Industrialization affected 0.99% of the area on agricultural land (t L) and 0.77% of the area on non-agricultural land (t). Increase of woody vegetation (a) affected 1.14% of the area. Other changes (oz) and changes smaller than 1.00% of the area affected 2.96% of the area.

Table 3. *Types of changes between the years 1974–1990*

Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)	TKind Type of change	Area (%)	Area (ha)
a	0.77	94.86	**i D**	1.89	232.24	**t**	0,73	89,58
a L	0.37	45.83	**O**	0.01	0.81	**t D**	0,04	5,26
bz	81.65	10,047.65	**O L**	0.02	2.98	**t L**	0,99	121,5
D	0.05	6.41	**oz**	1.27	156.03	**u**	2,36	290,22
e	2.33	286.12	**R**	0.38	46.29	**u D**	0,12	14,28
F	0.55	67.08	**R a**	0.02	2.55	**u L**	3,09	380,26
i	2.70	332.24	**R i**	0.49	60.55	**Z**	0,18	21,71

a - Increase of woody vegetation, **a L** – Increase of woody vegetation, Loss of agricultural land, **D** - Loss of woody vegetation, **e** - Extensification of agriculture, **F** - Forest management, **i** - Intensification of agriculture, **i D** - Intensification of agriculture, Loss of woody vegetation, **O** – Overgrowing, **O L** - Overgrowing, Loss of agricultural land, **R** - Remediation, Recultivation, **t** – Industrialization **i D** – Industrialization, Loss of woody vegetation, **t L** - Industrialization, Loss of agricultural land, **u** – Urbanization, **u D** - Urbanization, Loss of woody vegetation, **u L** - Urbanization, Loss of agricultural land, **oz** - Other changes/Unclassified

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Period 1990–2003

Between the years 1990 and 2003, 13.02% of the study area was changed and 86.98% of the study area remained unchanged. Information about the extent of all types of changes is in *Table 4*.

The most extensive type of change in this period was the intensification of agriculture (i) which affected 2.71% of the area. On the other hand, extensification of agriculture (e) affected 1.65% of the area. Increase of woody vegetation affected 2.34% of the area, mostly on agricultural land (a L). Changes to agricultural land were caused by the change of regime in 1989 and the following changes in ownership conditions. Changes in ownership conditions also caused remediation (R) on an area of 0.87%. Urbanization (u) affected 2.02% of the area and industrialization (t) affected 0.82% of the area. These changes were concentrated mainly at the end of this period, when the construction trends of big shopping malls and industrial parks began. Other changes (oz) and changes smaller than 0.50% of the area affected 3.48% of the area.

Table 4. *Types of changes between the years 1990–2003*

Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)
a	0.15	18.25	**i D**	0.19	23.37	**t**	0.34	41.85
a L	2.19	268.95	**O**	0.06	7.48	**t D**	0.01	0.95
bz	86.98	10,703.27	**O L**	0.24	29.81	**t L**	0.47	57.34
D	0.61	74.69	**oz**	0.76	93.95	**u**	0.48	58.65
e	1.65	202.61	**R**	0.55	67.34	**u D**	0.04	4.72
F	0.88	107.68	**R a**	0.06	7.41	**u L**	1.50	185.14
i	2.52	310.52	**R i**	0.26	32.54	**Z**	0.07	8.51

a - Increase of woody vegetation, **a L** - Increase of woody vegetation, Loss of agricultural land, **D** - Loss of woody vegetation, **e** - Extensification of agriculture, **F** - Forest management, **i** - Intensification of agriculture, **i D** - Intensification of agriculture, Loss of woody vegetation, **O** – Overgrowing, **O L** - Overgrowing, Loss of agricultural land, **R** - Remediation, Recultivation, **t** – Industrialization **i D** – Industrialization, Loss of woody vegetation, **t L** - Industrialization, Loss of agricultural land, **u** – Urbanization, **u D** - Urbanization, Loss of woody vegetation, **u L** - Urbanization, Loss of agricultural land, **oz** - Other changes/Unclassified

Period 2003–2015

Between the years 2003 and 2015, 9.08% of the study area was changed and 90.92% of the study area remained unchanged. Information about the extent of all types of changes is shown in *Table 5*.

This period is characterised by a significant loss of agricultural land due to the construction of industrial parks, big shopping malls and residential areas with family houses. The south town bypass (R1expressway) was also built in 2011. This is why the most significant kind of change was industrialization (t) which affected 2.58% of the area. Urbanization on agricultural land (u L) affected 1.77% of the area and urbanization on non-agricultural land (u) affected 0.88% of the area. Some of the areas lost their purpose and start to overgrow (O). This type of change affected 0.82% of the area. Other changes (oz) and changes smaller than 1.00% of the area affected 2.73% of the area.

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Table 5. *Types of changes between the years 2003–2015*

Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)	Type of change	Area (%)	Area (ha)
a	0.11	13.00	**i**	0.16	19.65	**t**	0.26	31.44
a L	0.13	15.63	**i D**	0.16	19.08	**t D**	0.08	9.38
bz	90.92	11,188.23	**O**	0.51	62.35	**t L**	2.51	308.34
D	0.23	28.02	**O L**	0.37	45.60	**u**	0.77	95.16
e	0.30	37.00	**oz**	1.18	144.98	**u D**	0.11	13.12
F	0.38	46.72	**R**	0.08	10.34	**u L**	1.77	217.31

a - Increase of woody vegetation, **a L** – Increase of woody vegetation, Loss of agricultural land, **D** - Loss of woody vegetation, **e** - Extensification of agriculture, **F** - Forest management, **i** - Intensification of agriculture, **i D** - Intensification of agriculture, Loss of woody vegetation, **O** – Overgrowing, **O L** - Overgrowing, Loss of agricultural land, **R** - Remediation, Recultivation, **t** – Industrialization **i D** – Industrialization, Loss of woody vegetation, **t L** - Industrialization, Loss of agricultural land, **u** – Urbanization, **u D** - Urbanization, Loss of woody vegetation, **u L** - Urbanization, Loss of agricultural land, **oz** - Other changes/Unclassified

Socio-economic development of the city of Nitra during the period 1949 to 2015

By 1949, the "building of socialism", era had begun, characterised by the nationalization of private property and creation of cooperatives. Small fields with different usage and owners were grouped into large fields owned by cooperatives. A diverse mosaic of agricultural crops were thus transformed into an intensively managed monoculture, grown over a large area. During this period, the economy was moved to central planning which greatly promoted the development of agriculture and industry in Nitra. In the outskirts of the city, objects of agricultural cooperatives and industrial plants were build. The Plastika Nitra factory was built in 1962 and was one of the largest industrial facilities in the city.

Between the years 1957 and 1967 new structures were built on areas gained by partial or overall sanitation of redundant objects. In the Sixties, the built-up area exceeded the original boundaries of the built-up part of the city, and the city began to grow beyond the river Nitra. The first structures built beyond the river were the College of Agriculture campus (now Slovak University of Agriculture) and the College of Education (now Constantine the Philosopher University). Later, the exhibition center Agrokomplex was built and put into operation in 1974. The construction of panel housing estates began in the Seventies. Chrenová – the first housing estate - was built beyond the river, followed by Klokočina and Diely, situated on the other side of the train line, and again these crossed the borders of the built-up area of the city. The extensive construction of panel housing estates was finished in 1992, while to a lesser degree the completion of individual residential houses has been continuing until now.

By 1989, significant socio-economic changes triggered by the Velvet revolution began as well as the collapse of the socialist establishment. The beginning of the 1990-ties is characterized by permanent changes in law, the level and structure of production and changes to prices and wages. The transformation of agriculture led to fundamental changes in the structure of agriculture in relation to new laws and rules of privatization and a massive restitution of land. After finishing the process of restitution, socialistic national cooperatives began to transform into cooperatives in private property. The majority of owners of restituted land are renting the land out at low prices to big companies (Bičík and Jeleček, 2009). Some

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of the owners of the restituted land divided the arable land into smaller areas intended for individual housing construction. Therefore, new town districts Šúdol, Prameň and Nový Lukov dvor (Nitra – Párovské Háje) were created.

Around the turn of the millennium, a new trend in urbanization, industrialization and the intensification of suburbanization had started. This process is connected to the construction of large-scale shopping malls, industrial parks, residential areas and related infrastructure. Construction takes place mostly on high-quality land resources, which leads to loss of fertile agricultural land (Bičík and Jeleček, 2009). The largest industrial park in Nitra is the Nitra-North Industrial Park, which will be fully occupied after the completion of the Jaguar Land Rover car factory and its subcontracted companies. The car factory will cover between 200 and 300 hectares in Nitra city and the adjacent municipalities Lužianky, Čakajovce and Zbehy.

In September 2011, the southern bypass of the city was finished, which is part of the R1 expressway that is part of the express connection between Bratislava and Banská Bystrica cities. Thanks to the R1 expressway, Nitra has in terms of car traffic very good connections with western and central parts of the Slovak Republic.

From 2010 to 2012 there was a slight deceleration of urbanization and industrialization, related to the financial crisis. After the consequences of the financial crisis subsided, and thanks to the inflow of foreign investment in industrial parks, the demand for land and apartments increased again. Urbanization is located mainly in the outskirts of the city, where there are mainly family houses and related infrastructure. On the other hand, mass housing construction consists mainly of brick apartment buildings situated on formerly unused areas within the built-up area of the city. Example are found in apartment complexes in the city district Zobor, in the housing estate Čermáň and in the former brewery in the old town. In July 2016, the remediation of the Polygon market started in order to build a multifunctional building (shops, offices, apartments...). Published in September 2016, was the intention to build a multifunctional building with flats ´Promenáda Living Park´ involving the reconstruction of the skeleton of the unfinished shopping center City Park near the river in the old town (the construction of City Park has been paused since 2007).

SWOT analysis

We prepared analysis of strong points, weak points, opportunities and threats (SWOT analysis) for the study area in three domains: social, economic and environmental sphere. The results are presented below.

Social sphere

SWOT analysis of social sphere is focused on evaluation of human resources, housing, social and health services, education, culture and sport in Nitra.

Strong points (S)
- high amount of skilled labor
- superior level of higher education
- low level of unemployment
- developed network of social housing
- approved community plan of social services in Nitra 2013-2018
- wide offer of social services

Weak points (W)
- negative demographic trends
- rising unemployment level of graduates of secondary schools and universities
- lack of rental housing
- insufficient capacity of facilities for seniors and disabled people (facilities

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- - existence of a University hospital and specialized ambulances - significant archeological findings, rich network of historic landmarks - existence of regional competence center for applied research and development in the field of agro-bio-technology (AgroBioTech) - wide offer of various forms of education - active sector of sport and physical education	providing residential, nursing and alternative services) - reserves in the protection and conservation of the cultural heritage of the city, poor technical condition of some sites, limited resources for the reconstruction of cultural heritage - insufficient promotion of the cultural offerings and cultural heritage of the city - unsatisfactory technical condition of buildings and equipment in certain educational institutions - lack of funds for the maintenance and equipment in schools - -lack of cooperation between the town and academia
Opportunities (O)	**Threats (T)**
- creation of new job opportunities for highly qualified labor and graduates of universities - support for families with children (financial contributions, housing, sufficient capacity of kindergartens...) - offer of housing for different income and social groups of citizens in cooperation with the private sector - existence of unused buildings suitable for housing, owned by the city of Nitra - increase of the quality of housing, renovation of housing stock - effective use of financial tools of EU structural policy - revitalization of unused and neglected areas for free-time activities - restoration of cultural heritage - better coordination and promotion of cultural activities and cultural heritage of the town - space for development of cycling and building of cycle routes	- leaving of skilled labor - negative demographic trends (decrease and aging of citizens) - rapid aging of the population and the related inability of providers of social and health services and residential facilities for seniors to adapt their capacity - the deterioration of the health status of the population - the deterioration of the social situation of inhabitants - increase in unemployment of risk groups - increase of number of maladjusted inhabitants - increase in crime - undersized financial resources for education - inaccessible social and "starter" apartments - unmanaged suburbanization - high-quality arable soil sealing - lack of funding - abuse of social system - disappearance of facilities for leisure activities - end of kindergartens, end or linking primary and secondary schools - residents not interested in the events in the town

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Economic sphere

SWOT analysis of economic sphere is focused on evaluation of economic development, tourism and traffic in Nitra town.

Strong points (S)

- favorable geographical location (proximity of borders, capital city and international airports in Bratislava, Vienna and Budapest)
- good connection to road network (R1)
- diversified economic base
- foreign investment (industrial parks)
- increasing intensity of business activity
- a lot of businesses focused on trade and services
- the existence of many scientific research institutions (Slovak Academy of Sciences, universities, National agricultural and Food Centre)
- tradition of exhibition (Agrokomplex)
- high number of historical monuments
- good traffic connectivity
- good conditions for wine tourism
- good conditions for incentive tourism
- reduction of traffic in the town center through construction of the southern bypass
- developed bus transportation (municipal, suburban and long-distance)

Weak points (W)

- low competitiveness of the labor market, lack of attractive job opportunities for highly skilled labor
- high unemployment level of university graduates
- rising share of long-term unemployed
- insufficient use of business potential opportunities in the cultural and creative sector
- loss-making exhibitions (Agrokomplex)
- insufficient promotion of the city
- insufficient supply of products for tourism
- low capital entry to tourism
- bad connection to rail network (location out of main railway routes)
- unsuitable railway connection
- missing integrated connection between rail and bus transport
- prolonged restoration of bus station
- bad technical state of the roads and pavements
- low attractiveness of municipal bus transport
- excessive preference of individual transport (cars)
- lack of parking spaces

Opportunities (O)

- ample free labor
- support of small and medium business
- preference towards industries based on new approaches and technologies
- connection of education and praxis
- expansion of existing and building of new industrial parks
- gaining of funds from EU programs
- investor interest
- support of residential and incentive tourism
- expanding and improving of services
- better city promotion

Threats (T)

- insufficient legal and financial support for small and medium business
- lack of resources for innovation
- ineffective programs focused on the labor market development
- low purchasing power of the population
- the departure of foreign investors and the emergence of "brownfields"
- preference towards new industrial buildings at the expense of arable land
- limitation on the possibility of

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- implementation of the potential of the town (exhibition, winery, history...) - increase in popularity of municipal bus transport - creation of integrated system of public transport (connection of municipal, railway and bus transport) - increasing interest in cycling - expansion of cycle paths and improvement of conditions for cycling - improvement of technical condition of communications - construction of communal car parks in housing estates	acquisition of resources from EU programs - "extinction" of town centre (relocation of shops and services to shopping malls, extinction of small shops) - lack of funds - stagnation in expanding and improving services - complete disappearance of vinery in the region - increasing of individual automobile traffic - poor coverage of area by municipal bus transport (low frequency of services, lack of bus-stops in the outskirts of the town, poor follow-up joints...) - public deem cycling unsafe

Environmental sphere

SWOT analysis of environmental sphere is focused on protected areas, waste disposal and quality of air, water and soil.

Strong points (S)	**Weak points (W)**
- existence of protected areas in the cadastre of Nitra - a high proportion of green areas in the city - established system of separate collection of waste (paper, glass, plastics, textiles, bio-waste) - functional urban composting - operation of 5 collection sites where citizens can submit waste for free - functional water treatment plant - approved Action Plan for sustainable energy in Nitra town - approved Program for improving quality of air for area of air quality management in urban area	- deficient protection and care for some protected areas (eg. Rolfesova mine) - position in the field of environmental hazard (Region 3 environmental quality) - pollution of surface water and groundwater - the existence of local pollution sources (e.g. illegal landfills) - Increased noise levels in some parts of the town (especially around the R1expressway) - rising degradation and loss of soil - close proximity of two nuclear power plants (Mochovce, Jaslovské Bohunice) and to the chemical factory (Duslo šaľa)
Opportunities (O)	**Threats (T)**
- improvement of the protection and management of protected areas in the cadastral area of the town	- extinction of protected plants or animals - rising of costs for waste disposal and

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- revitalization of watercourses and their surroundings - usage of renewable energy sources - elimination of local sources of pollution - revitalization and development of public areas - increase of the level of environmental awareness and behavior of town residents	recycling - increase in the amount of waste - continuance of degradation and loss of agricultural land, particularly in connection with new construction - lack of funding - absence or obsolete state of key policy documents for the creation and protection of environment

Scenarios of possible further development of the town

We elaborated scenarios of possible further development of the town based on information obtained from Planning documentation of the city of Nitra (ÚPN, 2003) and its additions, Program of economic and social development of Nitra (PHSR, 2016), SWOT analysis, official site of town (www.nitra.sk), press, news websites, analysis of the past land cover development and from our own field survey. Based on the evaluation of this information we decided to elaborate three scenarios: the best possible (progressive), the worst possible (negative) and balanced (realistic).

Negative scenario

In negative scenario, threats and weak points of the city have a strong influence on the development of the city. This variant expects slowdown or the end of economic growth, which can cause slowdown and a gradual end to industrialization and urbanization. There is a risk of bankruptcy of industrial factories and related formation of brownfields - abandoned and unused industrial areas. It is also expected that the trend of a decreasing and aging population will continue. With this trend a decrease of demand for housing and an increase of demand for social and health services is related (*Fig. 3*).

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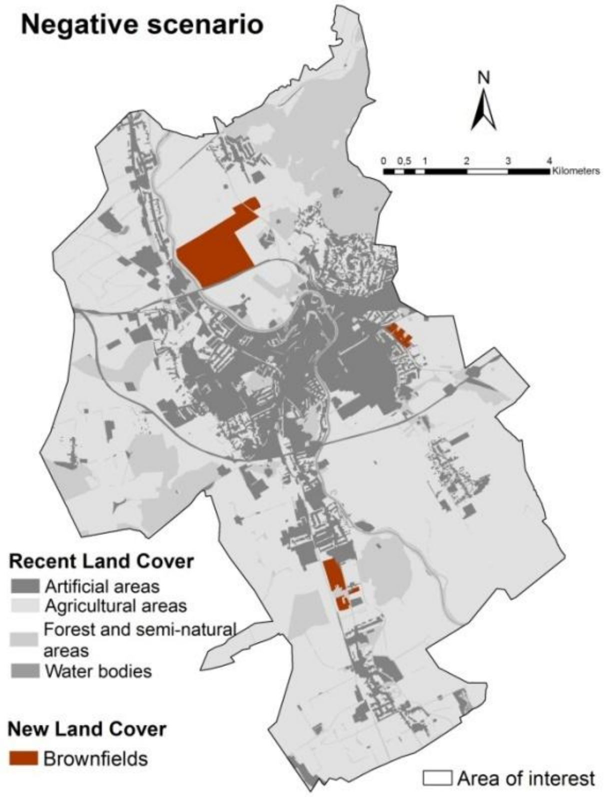

Figure 3. *Negative scenario of further development of the city of Nitra*

Progressive scenario

Progressive scenario expects strengthening of strong points and exploiting opportunities, which leads to rapid development of the city in all fields (favorable demographic trend and economic development) and a significant extension of urbanization and industrialization.

Based on this scenario it is possible to expect a significant extension of borders of the city whether due to impact of settlement area, construction of objects of trade and services, spreading of existing industrial facilities or due to the establishment of a new industrial park. This scenario expects a maximum approach to the environmental limits of the territory to achieve the most significant development of the city.

Progressive scenario anticipates filling to full capacity the Nitra-North Industrial Park in the cadastral territory Dražovce, and doubling the area of Nitra-South Industrial Park in the cadastral area Dolné Krškany. Near the Nitra-North Industrial Park there should be situated a newly established industrial park Nitra-West in the cadastral area of the village Lužianky. Additional industrial production is calculated in cadastres of the city parts Chrenová and Veľké Janíkovce.

New residents require expansion of accommodation capacities. Therefore, this problem is solved by the construction of a new housing development Párovské Lúky near the industrial park Nitra-North and by significantly spreading individual residential buildings in the outskirts of the city (*Fig. 4*).

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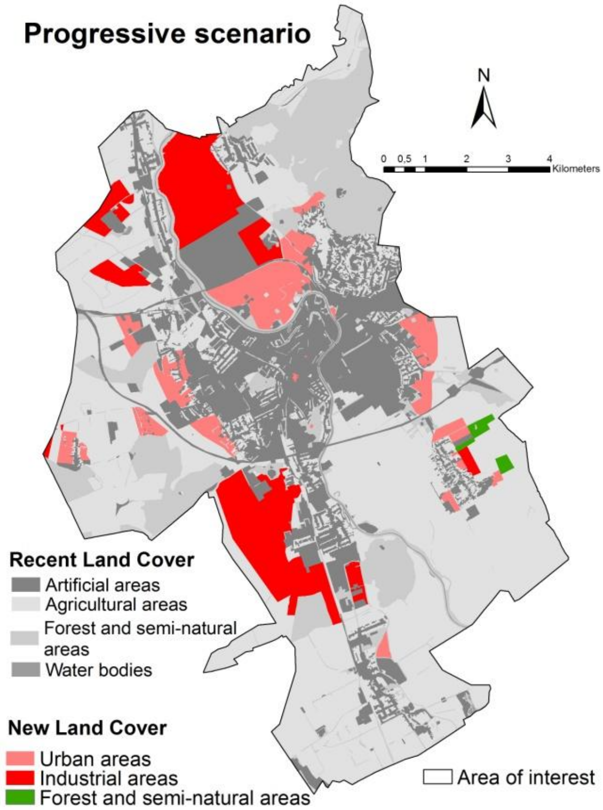

Figure 4. *Progressive scenario of further development of the city of Nitra*

Realistic scenario

This scenario aims to achieve a balance between the further development of Nitra and respecting the environmental limits of the area where the city is situated. It supports the development of the strong parts of the city and the exploiting of opportunities. On this basis, the expansion of urban boundaries can be assumed, but not as pronounced as in the case of the progressive scenario.

Realistic scenario is based on information about the confirmed investment (industrial sites, apartment complexes and mass housing development) and on the information gathered by field survey (the construction or sale of land for individual housing construction which has already started).

In housing, this scenario mainly focuses on the spread of individual housing areas on the outskirts of the city, it solves mass housing construction only by the construction of fewer apartment complexes within the urban area.

The capacity of the Nitra-North Industrial Park should be filled mainly thanks to the arrival of Jaguar Land Rover car factory and its subcontracted companies. New industrial factories can create a new Nitra-West Industrial Park situated in cadastral area of the village Lužianky. This new industrial park is connected with the existing Nitra-North Industrial Park and some factories situated on the outskirts of the village Lužianky. Also, several factories announced the spreading of their facilities, therefore the area of Nitra-South Industrial Park in Dolné Krškany should be expanded (*Fig. 5*).

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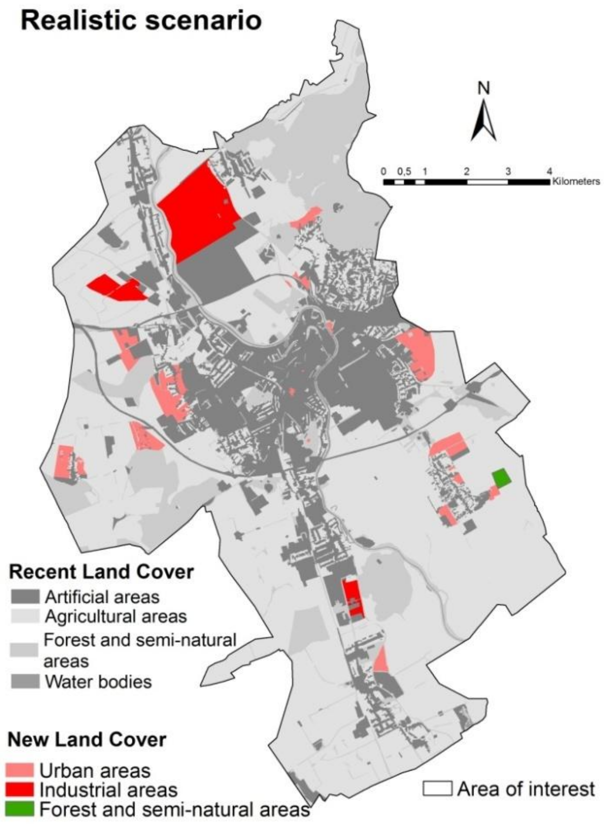

Figure 5. *Realistic scenario of further development of Nitra*

Discussion

Spreading of built-up areas is a natural consequence of city development. It is connected with processes of urbanization and industrialization that are accompanied by loss of agricultural land. For a development of the city that protects the environment maintains a good quality of life for its inhabitants, balanced management is needed. This management should be based on information from previous and recent city developments and the dispositions of areas for specific types of usage. This is why detailed mapping and evaluation of land cover and land use changes can be useful in creating good spatial planning documentation.

For modeling of future land use changes are the most commonly used mathematical modeling approaches like Markov Chain, SLEUTH, Dinamica EGO modeling with the Logistic Regression (LR), Regression Tree (RT) and Artificial Neural Networks (ANN) (Berberoglu et al., 2016). However, these approaches do not reflect changes of socio-economic situation or political (historical socialist planning) and economic changes. This is why we choose approach based on landscape-planning documentation of town, land use changes and our own field survey and created three scenarios of possible further development of the town. Each scenario reflects socio-economic and political situation in different era (see below).

For the future, we plan to verify changes in landscape use 5 years after the full operation of the car race and then use mathematic models to further prediction of landscape changes.

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The structure of land use changes has altered over the years. At the beginning of the period studied, the dominating changes were connected with agriculture production; at present, the dominating changes are connected with urbanization and industrialization. This variability was caused by significant political and economic changes such as “building the socialism”, the fall of socialism and following development of market economy. For the future, we plan to verify the changes in landscape use 5 years after the full operation of the car factory and then use mathematical models to further prediction of changes.

The development of industry brings a demand for labor, which has a positive impact on the migration of the population into Nitra. Nevertheless, the population has declined slightly, due to the low or negative natural population growth. This should be positively changed by the arrival of the Jaguar Land Rover car factory, other investors and the related rapid increase of job opportunities in Nitra and its vicinity. Closely related to the increase in job opportunities, is an increasing demand for housing. This demand can be satisfied by a new housing construction in the future. However, it will cause another increase of land use intensity in the studied area. The significant expansion of urban area, mainly due to the construction of the car factory, took place also in Trnava. Land cover and its changes in Trnava town were studied by Kopecká et al. (2015).

At the beginning of period studied, the extensive intensification of agriculture took place and borders of city did not change significantly. If the trend set in this period will continue in the future, it would match the negative scenario of city development. This scenario does not predict spreading of urban areas beyond the built-up areas of the city, it expects conservation and alternatively spreading of agricultural land.

In the Seventies, the construction of panel housing estates began and built-up areas of the city spread significantly. Construction of panel housing estates was ended in the Nineties, when the expansion of individual housing construction begun to prevail. Around the year 2000, the trend of constructing of large-scale shopping malls and industrial parks started. If the trend set in this period will continue in the future, it would match the progressive scenario of the further development of the city. It would bring a significant expansion to the urban area, sealing of agricultural soil and rapid development of industrialization.

At the end of the period studied, significant sealing of agricultural soil due to the construction of industrial parks is clearly visible. Recently, the construction of a car factory in the industrial park which is situated in the northeast part of the cadastral territory appears to be the most obvious change and impact on the landscape in Nitra. Industrial development can have both a positive and a negative impact (Kolejka and Klimánek, 2015). The economic situation appears to be positive because it will reduce the unemployment of the region (Lumnitzer et al., 2015), but in future, the land use change could be the negative. The impact can be identified as direct or indirect. The indirect impact of the automotive industry on the landscape is currently visible in the agricultural landscape. An increase of the biofuel content in fuels (Hammond et al., 2008) is reflected in the homogenization of growing crops. This can have a significant impact on the loss of biodiversity and ecosystem services of territories (Bourke et al., 2014; Scheper et al., 2015). The direct impact is related to the sealing and the land use of the most fertile parts of the agricultural landscape. Land use changes may lead to deterioration of water and soil quality (Dai et al., 2017) or loss of landscape character (Hreško et al., 2015; Li and Huang, 2015; Svobodová et al., 2015).

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A similar phenomenon was observed by Kopecká et al. (2015) in relation to the construction of the Peugeot-Citroën car factory in southeast of Trnava. The built-up area of Trnava has thus expanded by 332 ha. In Nitra, the construction of the Jaguar Land Rover car factory began in 2015. This factory will occupy arable land with an area of 200-300 ha. A significant loss of agricultural land was also observed due to the construction of the Kia car factory near Žilina.

Closely related to the increase in job opportunities, is an increasing demand for housing. The current trend of development corresponds to a realistic scenario of further development of the city, but it is inclining towards a progressive scenario due to the rapid spreading of industrial parks and an increased demand for housing.

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